a) STUDIES IN CHOLESTEROL METABOLISM.

b) STUDIES ON THE OXIDATION OF COLLOIDAL AQUEOUS SOLUTIONS OF CAROTENE.

by

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Thesis presented for the Degree of Ph. D.

University of Edinburgh.

May, 1944

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PART I

STUDIES IN CHOLESTEROL METABOLISM.

PART I

INTRODUCTION.

The following work was undertaken in the hope of throwing some light on the mechanism of gallstone formation with particular reference to the influence of diet on the concentration of cholesterol in the blood and bile.

The normal gall bladder concentrates the liver bile approximately ten times by the passage of water and inorganic salts through its wall. Bile pigments, bile salts and cholesterol are not absorbed to any appreciable extent so that gall bladder bile is considerably richer in these constituents than liver bile. It is undecided whether or not cholesterol is actually secreted by the normal gall bladder mucosa; most authors deny its occurrence although Elman & Taussig (1) present positive evidence for it. There is a pathological condition known as "strawberry gall-bladder" in which the mucosa becomes thickly impregnated with lipoid material containing a high proportion of cholesterol. Patey (2) reports experimental production of this condition in rabbits by

a combination of raising the blood cholesterol and inducing chronic inflammatory changes in the gall-bladder. This, however, cannot be taken as evidence for the secretion of cholesterol under physiological conditions and it is generally accepted that the high cholesterol content of gall-bladder bile is solely the result of concentration.

The more common varieties of gall-stones all contain a high proportion of cholesterol; bile pigments and calcium carbonate may also be present. Since cholesterol is usually the main constituent, a change in its behaviour is probably the primary factor in stone formation, and hence most worthy of consideration.

The mechanism of gallstone formation is not clearly understood, and the responsibility is attributed to various factors. The first of these is change in the bile salt; cholesterol ratio. Cholesterol is insoluble in water and in physiological saline, and hence must be kept in solution by some other constituent of the bile, which is usually assumed to be the bile salt fraction. A water-soluble bile salt-cholesterol complex

has been postulated, and Schøenheimer & Hrdina (3) report that precipitation of bile acids from bile by means of ferric chloride produces a simultaneous precipitation of cholesterol. Hence, if the concentration of bile salts should fall below a certain level the cholesterol will no longer remain in solution, and there are many reports that in cases of cholelithiasis the bile salt content of the bile is abnormally low. According to Hammarsten (quoted by Andrews & Hrdina (4)) the normal bile salt: cholesterol ratio is 40; if this falls below 13 the cholesterol is liable to be precipitated.

The lowered ratio may be due inter alia to decreased secretion of bile salts by the liver or to absorption of bile salts by the gall-bladder wall. In the event of liver damage there will be decreased bile salt production, but this will usually be accompanied by a proportionate decrease in cholesterol excretion, so that the ratio will not alter appreciably. Bacterial infection is thought to be mainly responsible for the loss of bile salts by the gall-bladder, the inflammatory changes in the mucosa rendering

it permeable to bile salts. This theory has been put forward to explain the frequency with which cholelithiasis follows cholecystitis, and Reinhold and co-workers report that nearly all gall-bladders which showed evidence of inflammatory changes contained calculi or crystals of cholesterol.

Dog bile is known to be an efficient solvent for human gallstones and this property is usually attributed to its high bile salt: cholesterol ratio (Pickens, Spanner & Bauman (5)). On the other hand Dolkart et al (6) maintain that the solvent action of dog bile is due to the fatty acid fraction rather than to the bile salts. The fatty acid fraction is considerably higher in dog than in human bile. Further doubt is cast on the view that bile salts alone are responsible for maintaining cholesterol in solution by the fact that in plasma the concentration of bile salt is very low, while that of cholesterol is relatively high.

However, neither the reduced bile salt production owing to liver damage nor the loss of bile salt through the inflamed gall-bladder wall can wholly account for stone

formation, as there is a significant proportion of cases of cholelithiasis in which the liver is normal and the bile is sterile. The small "mixed stones" containing cholesterol, pigment and calcium carbonate are usually associated with an infected gall-bladder, but the "cholesterol solitaire", a single stone almost entirely composed of cholesterol, seems to be formed independently of infection or injury of any sort.

It has been suggested that this type of stone is associated with hypercholesterolaemia, for example during the latter months of pregnancy, when gall-bladder disease is often first manifested. However, according to Gardner and Gainsborough (7) the total cholesterol of the plasma is not raised sigmificantly during pregnancy although there is an increase in the ratio between the free and ester forms. The fact that gallstones are rarely found in cases of lipoid nephrosis in which the blood cholesterol is exceedingly high (300 - 1000 mg. per 100 cc.) does not bear out the relationship between hypercholesterolaemia and cholelithiasis. And

further evidence against it is provided by Johnson & Riegel (8) who observed the effect of experimental hyper- and hypo-thyroidism on the concentration of cholesterol in the bile of dogs with total biliary fistulae. In none of the dogs was the cholesterol concentration or the total cholesterol output of the hepatic bile effected significantly, although the blood cholesterol was made to fall as low as 40 mg per 100 cc. and rise as high as 363 mg per 100 cc.

Prolonged stasis of the bile within the gall-bladder is also said to be a contributing factor in the formation of gallstones, mainly of the pigment-calcium variety with a very low content of cholesterol. However, Hrdina & Andrews (9) on the basis of their experiments on dogs, consider that stasis per se does not initiate the formation of calculi. The common bile ducts of large numbers of animals were ligated and in no case did prolonged stasis cause the appearance of stones or any change in the bile salt: cholesterol ratio.

The pH of the bile must also be of some importance since it is known that gall-

stones will dissolve in the acid bile of carnivorous animals but not in the alkaline bile of herbivors nor in the neutral bile of omnivors. Human liver bile is slightly alkaline (pH 8.2) and gall-bladder bile almost neutral, owing to loss of bicarbonate through the gall-bladder mucosa, so that any failure in the concentrating power of the gall-bladder will result in an alkaline gall-bladder bile which may encourage the precipitation of calcium carbonate. These crystals of calcium carbonate will act as nuclei around which the secondary precipitation of cholesterol and bile pigments will take place. The question of the pH of bile and its part in the aetiology of gallstone formation is fully discussed by Drury, McMaster & Rous (10), by Reinhold & Ferguson (11) and by Fieldman et al. (12).

There remains the question of the origin of the cholesterol in bile. Is it purely exogenous or is it synthesised either in the liver or at some other site in the body and excreted into the bile?

In animals there is definite evidence of cholesterol synthesis. Schøenheimer and Breusch (13) carried out cholesterol balance

experiments on mice and found definite synthesis on cholesterol-free diets (average synthesis per mouse per day was 1.8 mg). If moderate amounts of cholesterol were fed, the average synthesis was less (1.1 mg per mouse per day), and if the amount fed was great there was definite destruction of cholesterol. The results are confirmed by similar experiments carried out on rats by Channon (14). Furthermore, cholesterol is present in the eggs of birds when the diet contains little or none of that substance.

From the evidence in the literature it seems certain that cholesterol can be synthesised in the animal body if insufficient is provided in the diet and destroyed if it is in excess, and it is probable that this state of affairs also holds in the human body. Certainly it would appear that the small amount in the normal mixed diet would be insufficient to supply the large body reserves of cholesterol. This view is supported by the balance experiments of Gardner & Fox (15) and others which showed that the sterol excreted in the faeces invariably exceeds that absorbed in the food.

The following experimental work was designed to investigate the effect of dietary cholesterol on the blood and bile cholesterol. In the past it has been widely believed that the level of cholesterol in the blood and bile is influenced by the intake of cholesterol in the food and for this reason the diet of patients with disorders of the biliary system has been restricted to foods low in cholesterol. The results of this work suggest that this irksome restriction may be unnecessary, since the concentrations of cholesterol in the blood and bile do not appear to be significantly affected by the ingestion of comparatively large amounts of that substance.

METHODS.

At the beginning of this work the estimations of cholesterol in plasma from oxalated blood were made by Myers and Wardell's adaptation of the Liebermann-Burchard colorimetric technique (16). However, this method was not found to be entirely satisfactory, and as it could not be applied to the estimations in bile owing to the presence of the pigments, the digitonin-precipitation method of Okey (17) was adopted. This technique is considerably longer and more tedious, but it has the advantage of distinguishing between the free and esterified forms of cholesterol. In addition, it can be used for the analysis of any biological fluid. Estimations of the free cholesterol and of the total cholesterol (after saponification) were carried out in duplicate and good agreement was obtained. The figures for the ester form were calculated by difference.

1. Effect of High- and Low-Cholesterol Diets on the Level of Cholesterol in the Plasma.

There are many conflicting reports in the literature on this point. In fact there is no agreement as to the constancy or variability of the blood cholesterol level in the same individual over a considerable period of time. Sperry (18) determined the total cholesterol in the blood serum of healthy adults over varying periods of time up to 28 months and concluded that there is a constitutional level which is characteristic for each individual and from which large deviations do not ordinarily occur. Gildea, Kahn & Man (quoted by Sperry) confirm this conclusion; whereas Okey and Stewart (19) found considerable variability in the blood cholesterol level, particularly in women (apparently connected with the menstrual cycle). Gardner & Gainsborough (20) and McEachern & Gilmour (21) are in agreement that there is a striking fluctuation from hour to hour in the blood cholesterol of a single individual, and that these variations cannot be due to absorption of the substance.

Gardner and Gainsborough (22) state that the cholesterol concentration of human

plasma can be raised or lowered slightly by sufficiently prolonged feeding with diets of high (1.4 gms. cholesterol per day) or low (60 mg cholesterol per day) sterol content. These changes are most marked in the ester form, the free cholesterol remaining practically constant. According to McEachern and Gilmour (23), the cholesterol in the food has very little effect on the blood level unless the high sterol diet is prolonged over a considerable period of time. They did, however, significantly increase the cholesterol of the blood by feeding this substance to persons with cholecystitis and cholelithiasis.

Okey and Stewart (20) state that there is a consistent rise in blood cholesterol on a high cholesterol diet, but they also report a great daily variability in the absence of dietary changes.

Patey (24) fed rabbits on 1 gm. of cholesterol daily for two months and demonstrated a ten-fold increase in the concentration of sterol in the plasma. Cholesterol is, however, an unnatural food for rabbits and the amount ingested, relative to body weight, was enormous.

On the whole, the conclusion to be drawn from the literature is that whilst it is possible to alter the plasma cholesterol by large alterations in the cholesterol intake, ordinary "cholesterol rich" diets do not significantly raise the plasma cholesterol above the level found when "low cholesterol" diets are eaten, or at least do not produce an increase sufficient to affect the cholesterol content of the bile.

Experimental Part.

Hospital patients were fed on low-cholesterol diets (containing about 300 mg. sterol per day) and blood specimens were analysed for cholesterol at intervals of three days, all blood samples being taken at the same time of day, i.e. 10.30 a.m., 2½ hours after the last meal. Then the cholesterol content of the diet was increased by the addition of 900 mg. of pure cholesterol per day, three eggs (approximately 900 mg. of cholesterol per day) or 50 gms. of sheep's brain (approximately 2,000 mg. cholesterol per day), the cholesterol content of the blood again being determined at three-day intervals on samples taken at the same time of day as

during the previous period. The results are shown in Table I.

In cases 6 and 10 there is a distinct rise in the level of cholesterol in the plasma after the ingestion of either the crystalline substance or eggs for a period of three days and cases 2 and 9 show a rise on the diet containing pure cholesterol although this is not maintained in case 9 when the sterol is provided in a diet of eggs. And, whereas case 2 shows a significant fall in plasma cholesterol concentration after the standard low cholesterol diet has been given, cases 3 and 8 actually show a rise in the same circumstances. Of the other five subjects, none shows any striking decrease or increase in plasma cholesterol which can be directly related to the diet. There is evidently a variation due to endogenous causes.

Ten other cases, in which the concentration of cholesterol in the plasma was determined by the digitomin-precipitation method of Okey, are recorded in Table II.

In case 8 the high cholesterol diet is associated with a definite increase in the concentration of esterified cholesterol

TABLE I

CASE	CONDITION OF PATIENT.	PLASMA CHOLESTEROL AFTER DIET A MG/100cc.	PLASMA CHOLESTEROL AFTER DIET B MG/100cc.	PLASMA CHOLESTEROL AFTER DIET C MG/100cc.	PLASMA CHOLESTEROL AFTER DIET D MG/100cc.
1	Acute gall-bladder colic. No operative procedure.	135	140	155	-
2	Cholecystitis. No operative procedure.	152	132	151	-
3	Catarrhal jaundice. Obstruction of C.D.	102	122	107	-
4	Biliary fistula. Jaundice.	155	146	139	150
5	Cholelithiasis. Biliary fistula. Jaundice.	97	101	100	107
6	Bronchial asthma.	144	151	171	215
7	Lung abscess.	206	211	184	216
8	Asthma.	142	167	155	161
9	Lymphadenoma.	140	134	185	148
10	Acute cholecystitis.	145	150	191	199

DIET A = ordinary ward diet before experiment was begun: low in cholesterol.
DIET B = standard low-cholesterol diet containing approx. 300 mg. cholesterol per day.
DIET C = " " " " supplemented by 300 mg. of crystalline cholesterol three times a day, i.e. it contained approx. 1,200 mg. per day.
DIET D = " " " " supplemented by 3 eggs per day, i.e. it contained approx. 1,200 mg. cholesterol per day.

Diets B,C & D were, in each case, maintained for a period of three days, before the plasma analyses were made. The total cholesterol concentration of the plasma was estimated by the colorimetric method, duplicate analyses being made in each instance; the figures recorded are the mean of these duplicate readings.

TABLE II

CASE	CONDITION OF PATIENT.	PLASMA CHOL. AFTER DIET A MG/100cc.			PLASMA CHOL. AFTER DIET B MG/100cc.			PLASMA CHOL. AFTER DIET C MG/100cc.			PLASMA CHOL. AFTER DIET D MG/100cc.			PLASMA CHOL. AFTER DIET B 1 MG/100cc.		
		Free	Total	Ester	Free	Total	Ester	Free	Total	Ester	Free	Total	Ester	Free	Total	Ester
1	Gaucher's disease. Splenectomy four months previously.	75.2	176.0	101.0	70.5	169.0	98.5	-	-	-	82.8	190.2	107.4	-	-	-
2	Cystocele and Rectocele.	68.8	178.4	109.6	66.6	177.2	110.6	-	-	-	69.0	183.0	114.0	-	-	-
3	Rheumatoid arthritis.	69.8	166.4	96.6	77.9	179.5	101.6	-	-	-	73.1	170.5	97.4	-	-	-
4	Biliary fistula. Gross jaundice.	65.8	176.9	111.1	51.1	171.0	119.9	-	-	-	56.0	172.5	116.5	-	-	-
5	Biliary fistula. No jaundice.	48.7	153.4	104.7	-	-	-	-	-	-	42.2	167.6	125.4	58.3	170.5	112.2
6	Cerebral thrombosis.	73.1	158.3	85.2	59.1	112.0	52.9	-	-	-	53.5	138.0	64.5	63.3	103.7	40.4
7	Biliary fistula. No jaundice.	57.6	162.7	105.1	81.7	140.5	58.8	-	-	-	69.5	165.5	96.1	73.6	156.9	83.3
8	Hypertension.	72.1	171.5	99.4	62.3	174.1	111.8	75.2	220.5	145.3	-	-	-	51.0	155.4	104.4
9	Hypertension.	83.6	240.1	156.5	96.6	269.0	173.6	69.3	214.1	144.8	-	-	-	76.5	220.6	144.1
10	Biliary fistula. No jaundice.	62.3	175.9	113.6	70.5	178.0	107.5	-	-	-	61.6	183.1	121.5	-	-	-

The diets are the same as given for Table I except that sheep or ox brain was substituted for the eggs, owing to difficulty in obtaining the latter.

DIET D = standard low-cholesterol diet supplemented by 50. gms. brain (cooked) giving a total cholesterol intake of about 2,300 mg. per day.

DIET B 1 = return to standard low-cholesterol diet for three days after previous period of three days on high-cholesterol diet.

in the plasma, which falls on the return to the diet of low cholesterol content; in Case I there is a slight increase both in the free and ester forms which can be related to diet. The same is also found in Case 5 although the increase is still maintained on subsequent return to the standard low-cholesterol diet. In no other subject can a change in the level of cholesterol in the plasma be directly related to a change of diet, and hence the foregoing results would indicate that the blood cholesterol is not directly dependent on the intake of that substance, at least within the periods covered by the experiments, which could not in every instance be continued as long as was desired, owing to the clinical condition of the patient.

NOTE. The cholesterol content of the diets was calculated from the values in Stern's "Applied Dietetics" and is approximate. However, it is thought that the difference between the low- and high-cholesterol diets is sufficiently great to render slight inaccuracy in calculation of sterol content unimportant.

A few cholesterol analyses were carried out on eggs and brain by the digitonin-precipitation method. The foodstuff was ground up thoroughly with silver sand and extracted overnight in a Soxhlet apparatus, with ether as the solvent. The analysis was carried out on an aliquot of the ether extract. The results are shown overleaf.

FOODSTUFF.	MG. CHOLESTEROL/100 GMS.
Egg (whole, hen)	1. 486 2. 520 3. 682 4. 590 5. 710
	Average 599 mg. cholesterol/100 gms. egg.*
Brain (cooked)	1. 3830 2. 4020 3. 4710 4. 3443 5. 3910
	Average 3983 mg. cholesterol/100 gms. brain.

* The average weight of an egg is 50 gms. Hence, one egg is equivalent to about 300 mg. cholesterol.

2. Effect of Ingestion of a Single Dose of Cholesterol on the Concentration of Cholesterol in the Plasma.

Gardner and Gainsborough (22) report that alimentary hypercholesterolaemia does not occur and that there is no connection between the amount of sterol ingested and the cholesterol level of the plasma during digestion. Although changes frequently occur, sometimes an increase and sometimes a decrease compared with fasting values, the authors consider these changes evidence of an active endogenous metabolism in which cholesterol takes part during the process of digestion.

Knudson (25) investigating the effect of ingested fat (free from sterol) on the blood cholesterol of dogs which had been fasting for 24 hours prior to the experiment, found that although the total cholesterol in blood remained fairly constant during fat absorption, the cholesterol esters increased, reaching a maximum about the 6th hour. The same author (26), again using dogs previously fasted, tried the effect of a fat-free meal supplemented by 2.5 - 4 gms of cholesterol or cholesterol esters and found always an increase in the total cholesterol of the blood, the peak usually occurring about the

sixth or eighth hour after the meal. In these experiments there was no increase in the amount of esterified cholesterol.

Fitz and Bruger (27) report slight increments in the esterified cholesterol of the blood after ingestion of large doses (100 gms) of glucose.

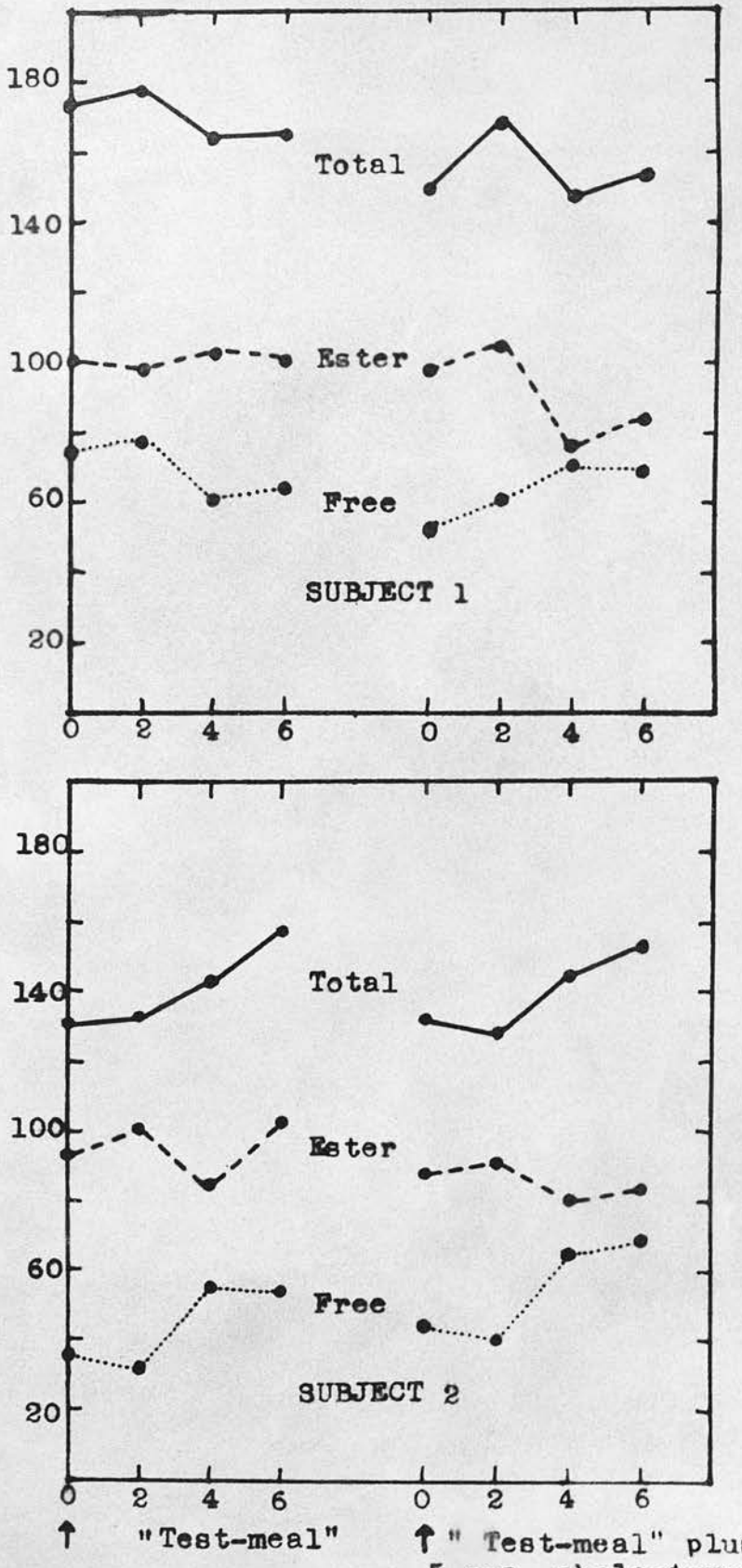
McEachern and Gilmour (21) concluded that it was impossible to plot a standard normal blood cholesterol curve owing to the striking fluctuations from hour to hour in the blood cholesterol (156 - 170 mg.%). They also fed normal subjects on as much as 2 - 10 gms. of pure cholesterol suspended in milk and found no significant evidence of cholesterol absorption into the blood.

The weight of the above evidence seems to favour the view that ingestion of a single dose of cholesterol has no direct constant effect on the blood cholesterol, a conclusion which is supported by the following experiments.

Experimental Part.

Four normal subjects who had fasted for 24 hours prior to each experiment were given a "cholesterol-free", "fat-free" meal, consisting of porridge, toast, marmalade and tea or coffee

FIG. I


180
140
100
60
20
Total
Ester
Free
SUBJECT 1
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180
140
100
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20
Total
Ester
Free
SUBJECT 2
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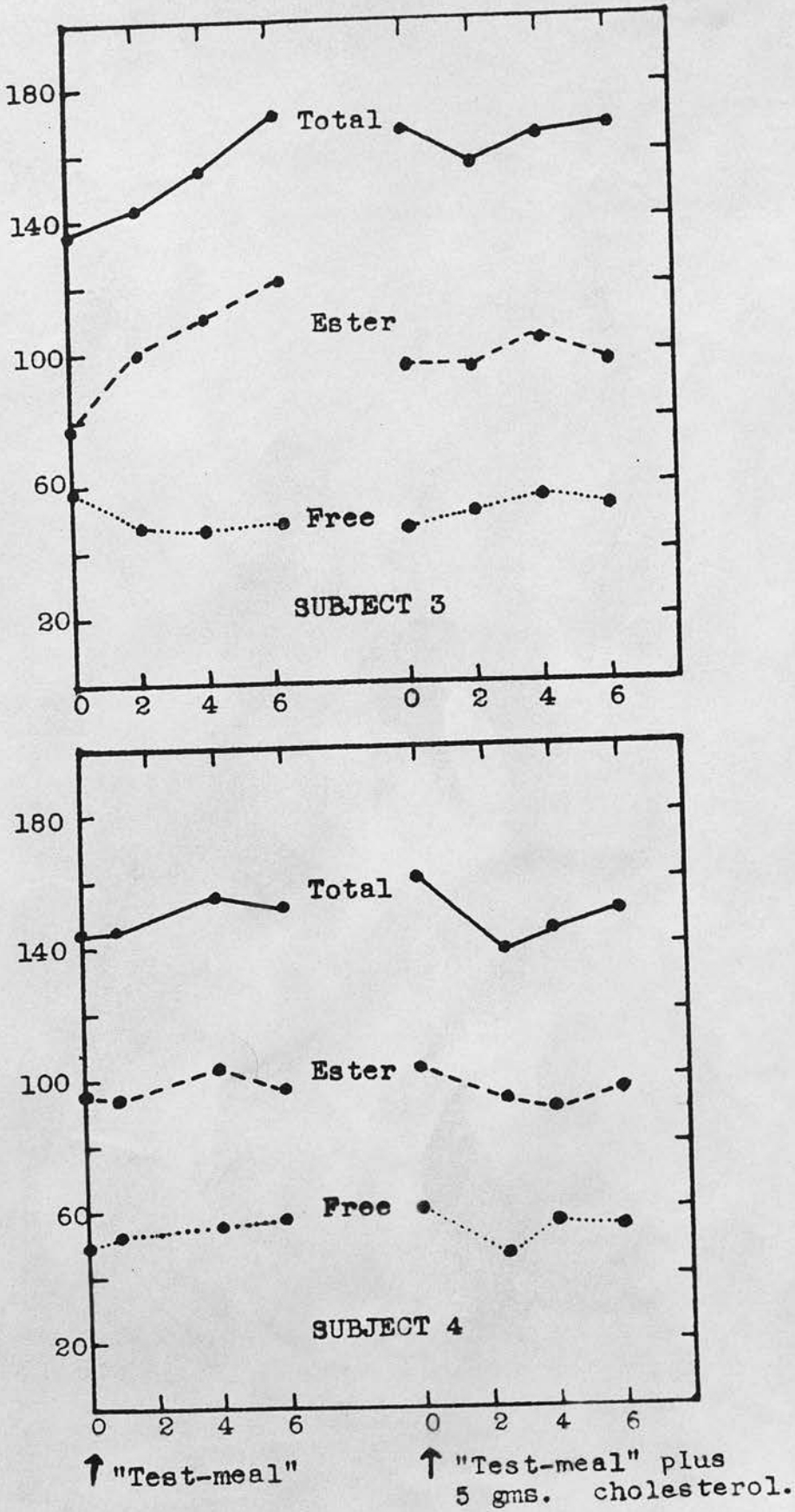
"Test-meal"
" Test-meal" plus
5 gms. cholesterol.

FIG. II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Total
Ester
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SUBJECT 3
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2
4
6
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2
4
6
180
140
100
60
20
Total
Ester
Free
SUBJECT 4
0
2
4
6
0
2
4
6
"Test-meal"
"Test-meal" plus
5 gms. cholesterol.

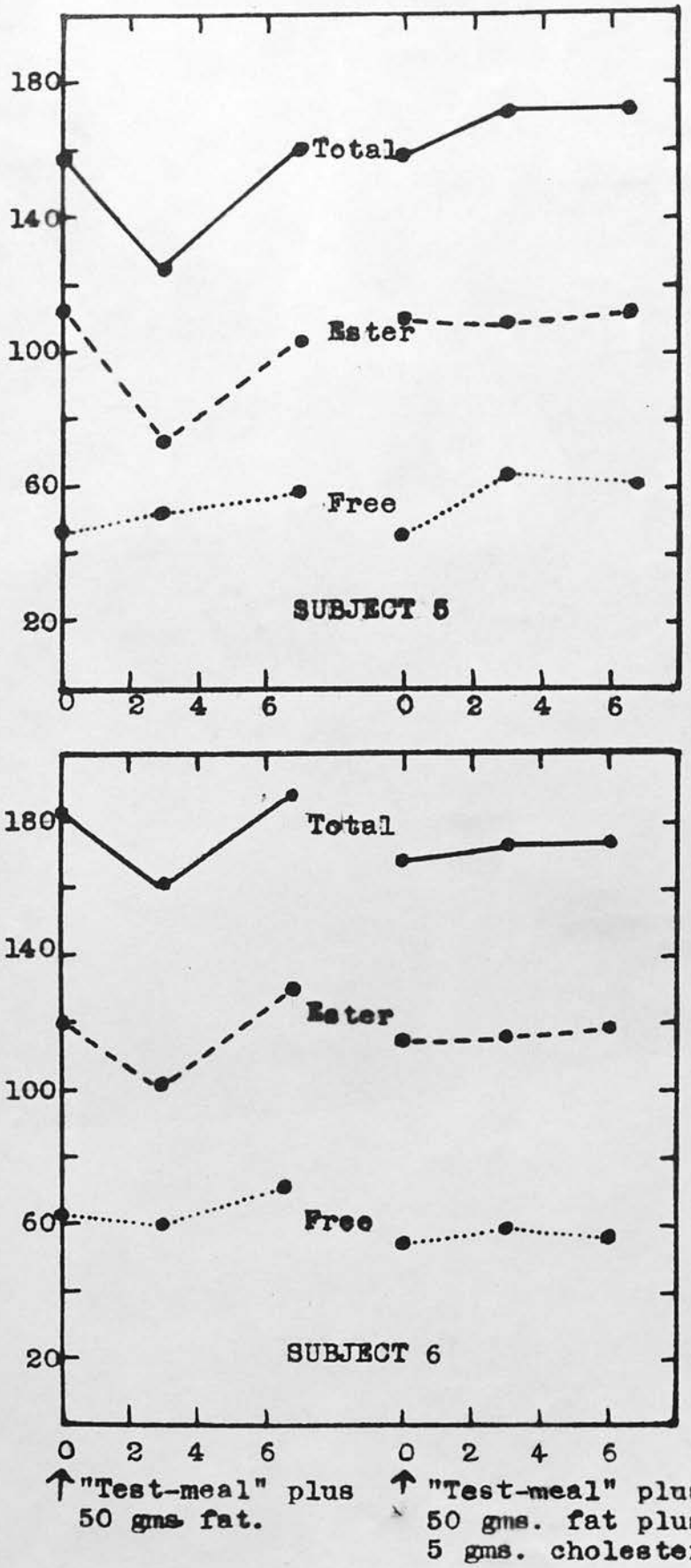
without milk. This meal was supplemented by 5 gms. of crystalline cholesterol, and plasma cholesterol curves were constructed over a period of six hours. These curves did not differ significantly from those obtained from the same subjects when given a similar meal without the added cholesterol.

The estimations of free and total cholesterol were done by the digitonin-precipitation method of Okey, and the esterified fraction computed by difference. The points on the graphs represent the mean of duplicate analyses. (Figs. I and II).

It is seen from Figs I and II that there is no consistent rise in the plasma cholesterol after ingestion of a relatively large quantity of the pure substance. There is a transient rise in the case of Subject I, compared with the control curve, but this is not repeated in any of the other three subjects. There is, in every case, considerable fluctuation in both the free and esterified forms of cholesterol.

Since it is possible that crystalline cholesterol is poorly absorbed in the absence of fat, these experiments were repeated with

FIG. III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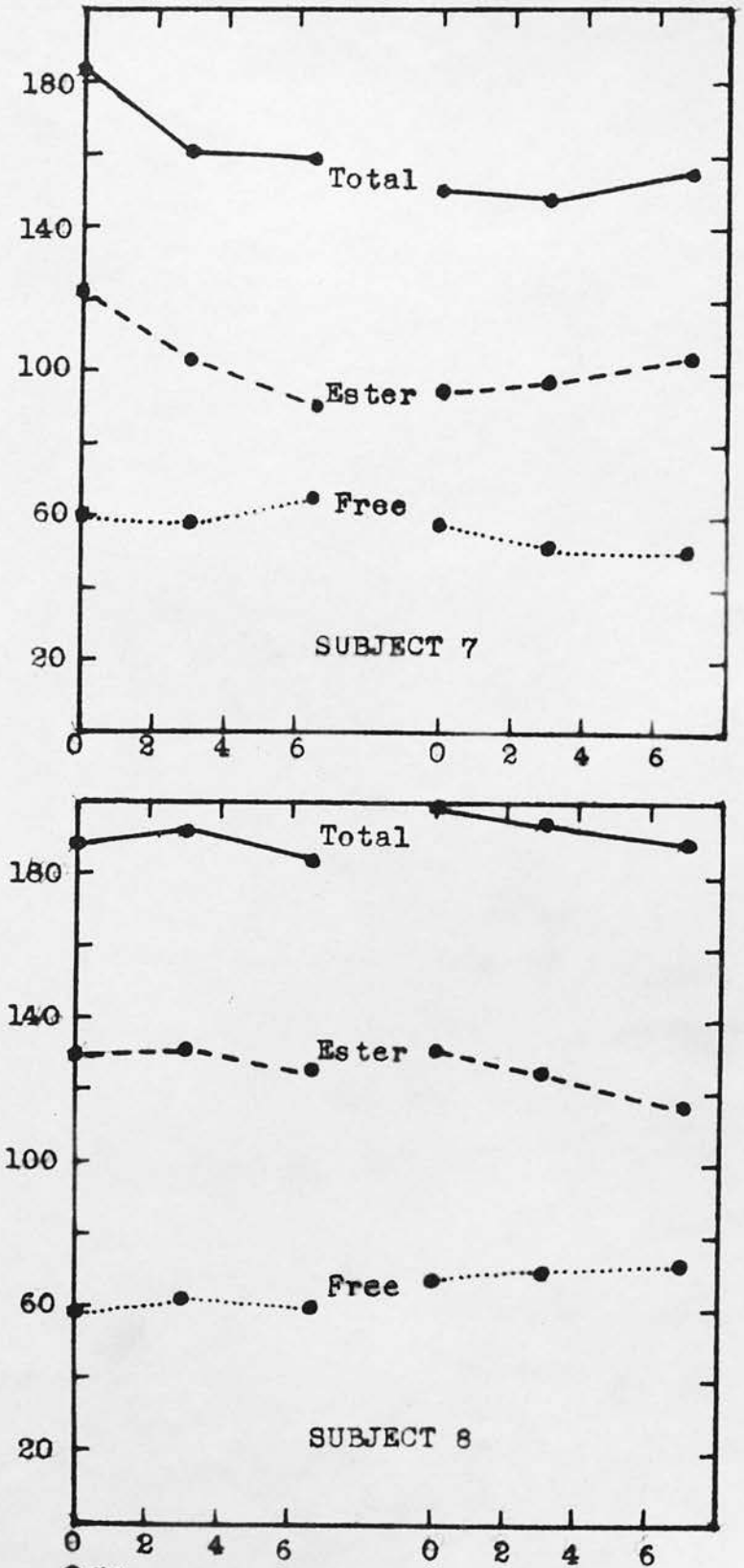
100
60
20
Total
Ester
Free
SUBJECT 5
0 2 4 6 0 2 4 6
180
140
100
60
20
Total
Ester
Free
SUBJECT 6
0 2 4 6 0 2 4 6
"Test-meal" plus 50 gms fat.
"Test-meal" plus 50 gms. fat plus 5 gms. cholesterol.

FIG. IV


180
140
100
60
20
Total
Ester
Free
SUBJECT 7
0 2 4 6 0 2 4 6
Total
Ester
Free
SUBJECT 8
↑ "Test-meal" plus 50 grms fat.
↑ "Test-meal" plus 50 grms. fat plus 5 grms. cholesterol.

the addition of 50 gms. of margarine to the "test-meal". Margarine was used since it is cholesterol-free. The striking feature of these results (represented in figs. III & IV) is that in three out of the four subjects (5, 6 and 7) there is a significant decrease in the concentration of esterified cholesterol in the plasma after the ingestion of fat, and this decrease does not occur when cholesterol is fed together with the fat. This observation is a direct contradiction of the findings of Knudson (see above). In no case, however, is there an increase in the plasma cholesterol concentration, either free or esterified, after the ingestion of the pure substance, and in this respect the results bear out the conclusions of Gardner and Gainsborough, quoted above, that the fluctuations in plasma cholesterol are evidence of an active endogenous metabolism in which cholesterol takes part during the process of digestion and are not directly connected with the ingestion of cholesterol. On the other hand, may not these fluctuations,which are quite irregular, merely be responses to changes in the physico-chemical factors which govern the solubility of cholesterol

in plasma? These factors are evidently complex. If Gardner and Gainsborough were correct the fluctuations, though unrelated to cholesterol ingestion, should at least be consistent.

3. Effect of High- and Low-Cholesterol Diets on the Cholesterol Content of Human Bile.

Most of the literature on this point relates to animal experiments and there is little information about the direct effect of diet on the concentration of cholesterol in human bile.

Whipple (28) states that the cholesterol content of human bile varies within wide limits and one can determine no uniform relationship to disease but that most authors are agreed that the commoner forms of liver damage are associated with a very low concentration of cholesterol in the bile, which suggests that the biliary excretion of this substance is in some way linked with the physiological activity of the liver.

Reinhold (29) and Riegel, Ravdin & Rose (30) are in agreement with the above statement that the cholesterol content of human bile varies widely, both in different patients and also from day to day in the same patient. On the other hand, Kohlstaedt & Helmer (31) found the composition of human fistula bile to be remarkably constant in spite of a variable diet, although it was possible by the oral administration of bile salt to alter the bile

salt - cholesterol ratio of the bile secreted by the liver, since the increased output of bile salt is not accompanied by a proportionate increase in cholesterol.

According to Riegel and co-workers (30) there is no relationship between the cholesterol concentration of the bile and fluid intake or amount of external drainage in patients with biliary fistulae. Re-feeding of the bile was without effect on the biliary sterol output.

Dostal and Andrews (32) on the basis of observations on a mild diabetic whose common bile duct had been intubated, concluded that the responsibility for gallstone formation lies with the gall bladder rather than with the liver. They observed no effect on the biliary cholesterol of feeding pure cholesterol either alone or together with bile salts. The same results were obtained by Stadelman (quoted by Whipple).

It seems that the only factor which has a constant and predictable effect on the bile cholesterol concentration is the presence of liver damage, and opinion favours the view that the marked fluctuations are independent of the intake of sterol in the diet.

Experimental Part.

Most authors agree that there are serious difficulties in the investigation of bile by the duodenal tube method owing to dilution by pancreatic secretion, etc. Hence, experiments were carried out only on patients with biliary fistulae. It has been possible to investigate ten cases, all patients in the Royal Infirmary, Edinburgh.

In four of these cases the clinical condition of the patients did not permit the giving of experimental diets and they were therefore kept on a light ward diet, of which the cholesterol content would rarely exceed 300 mg. per day. The remaining six cases were given a special low-cholesterol diet containing about 300 mg. cholesterol per day. After a varying period (see Table III) the diet was changed to one having a high sterol content, given as crystalline cholesterol or as brain, for a further period of three days.

Bile was collected from the fistulae on alternate days, and the cholesterol concentration determined by the digitonin-precipitation method. It is generally assumed that all the cholesterol in bile is present in the

free state, although Riegel, Ravdin & Rose (33) report that under rare conditions cholesterol may occur in human liver bile in the combined form. On the few occasions in the course of the present work when cholesterol was determined before and after saponification none of the ester form was detected. The presence of a cholesterol esterase in bile has been postulated to explain the absence of the esters.

The cholesterol content of the bile was invariably very low immediately after the operation but rose gradually, presumably as the liver damage lessened, although there was still considerable day to day variation.

This variation was most marked in a case with severe hepatic damage characterised by gross jaundice (see Fig. V). There was also a great daily variation in the volume of bile which drained externally. Cholecystectomy and choledochostomy had been performed on this patient five days previous to the experiment. She was exceedingly jaundiced. Throughout the duration of the biliary fistula the patient's condition did not permit the giving of experimental diets, but she had a poor appetite and very little fat was eaten, so that in consequence the diet was virtually sterol-free. No choleretics

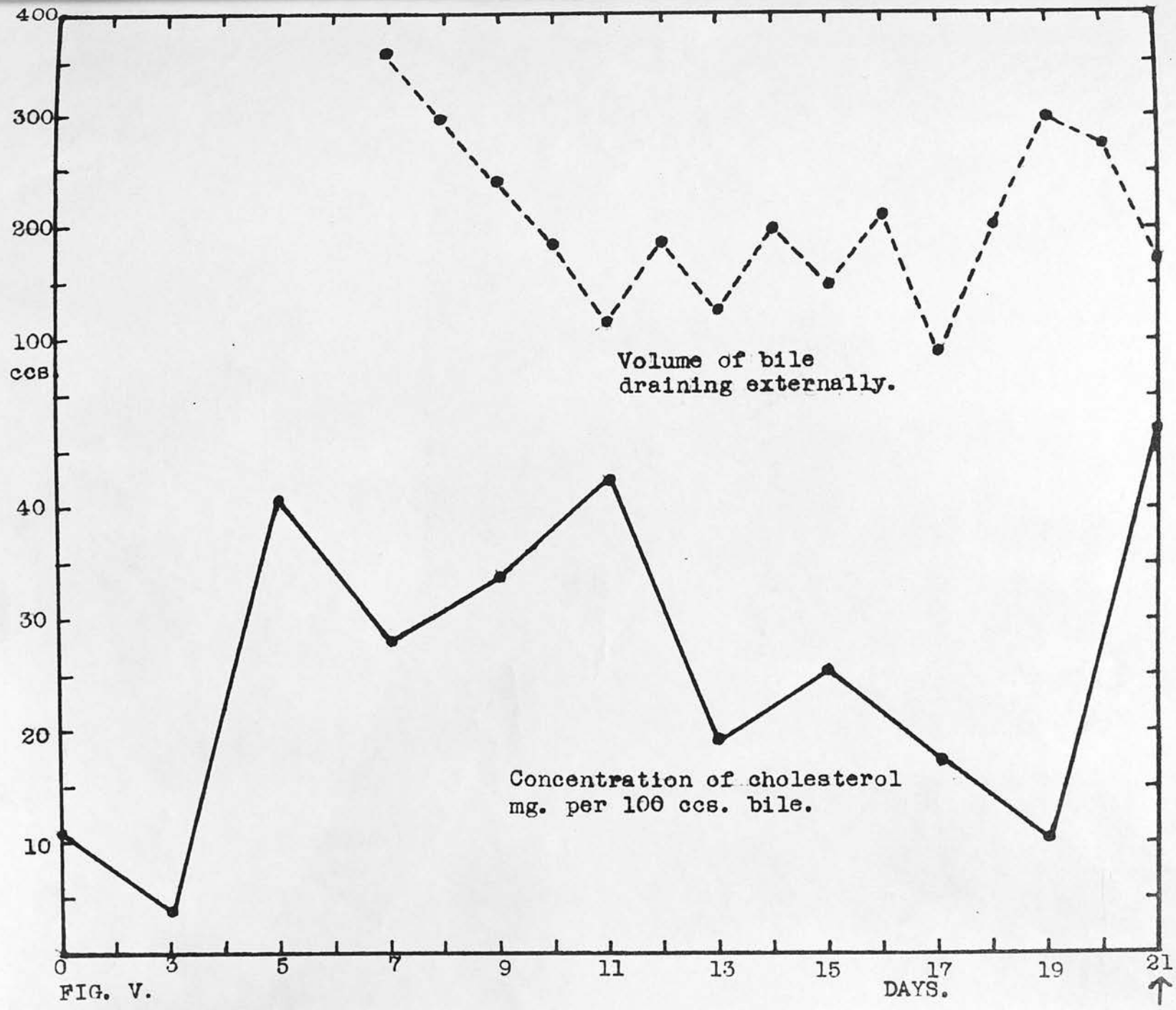

400
300
200
100
ccs.
40
30
20
10
Volume of bile
draining externally.
Concentration of cholesterol
mg. per 100 ccs. bile.
0
3
5
7
9
11
13
15
17
19
21 Fistula
closed.
DAYS.

FIG. V.

were adminstered. The plasma cholesterol was rather high. Two samples of blood were obtained, with a time-interval of three days.

Plasma Cholesterol Concentration
MG/100cc.

	Free	Total	Esterified
1st Sample:	104.7	284.9	180.2
2nd Sample:	97.4	242.5	165.1

In human beings with a biliary fistula it is impossible to determine what proportion of the bile is draining externally and what proportion is draining into the intestine via the common duct. Hence no calculation can be made of the total secretion of the bile or of cholesterol. But it is possible to compare the concentration of cholesterol in a given sample of bile with the cholesterol ingested in the diet.

Of the ten cases studied, one showed a distinct rise in the bile cholesterol concentration on a diet having a high sterol content (about 2 gms cholesterol per day given as 50 gms sheep's brain); one showed a rise when the high sterol diet was first given with a subsequent return to the original level (the patient still on the high diet); and in the other eight cases

no significant or sustained changes in the bile cholesterol concentration coincided with changes in the cholesterol content of the diet. Unfortunately, the duration of these experiments was limited by the short period of time over which the fistulae remained open.

The first six cases are compiled in a single table (Table III). The remaining three are recorded separately as the fistulae were maintained for a longer period of time enabling a greater number of analyses of the bile to be carried out.

TABLE III

CASE	CONDITION OF PATIENT.	PERIOD OF TIME BETWEEN OPERATION & COMMENCEMENT OF EXPERIMENT.	BILE CHOL. CONCENTRATION ON DIET A. MG/100cc.	BILE CHOL. CONCENTRATION ON DIET B MG/100cc.	BILE CHOL. CONCENTRATION ON DIET C. MG/100cc.	BILE CHOL. CONCENTRATION ON DIET D. MG/100cc.
1.	Cholecystectomy. Clinical condition good.	14 days.	Day 1 137 " 2 140 " 3 116	Day 4 111 " 5 99 " 6 80	-	-
2.	Obstruction of C.D. Biliary fistula of 2 mths. duration. Grossly jaundiced.	2 months.	20	24	27	-
3.	Stones in G.B. Biliary fistula. Grossly jaundiced.	11 days.	35	30	37	-
4.	Biliary fistula. Grossly jaundiced.	8 days.	Day 1 21.0 " 4 22.6	-	-	25.2
5.	Biliary fistula. No jaundice.	12 days.	Day 1 39.9 " 4 66.2	-	-	82.0
6.	Biliary fistula. No jaundice.	17 days.	Day 1 79.9 " 4 58.0 " 7 39.5	-	-	43.5
7.	Biliary fistula. No jaundice.	22 days.	Day 1 52.5 " 3 62.3 " 5 48.6	-	-	51.7

DIET A = low-cholesterol diet containing less than 300 mg. cholesterol per day.
DIET B = " " " supplemented by 900 mg. cholesterol per day.
DIET C = " " " " " three eggs (900 mg. cholesterol per day).
DIET D = " " " " " 50 gms. brain (2000 mg. per day)

Unless otherwise stated, patients were given the experimental diets for three days before a sample of bile was taken for analysis.

In case 1 the cholesterol concentration of the bile was higher than normal, which is 50 - 80 mg.%, but it progressively decreased in spite of the oral administration of pure cholesterol in an amount greater than that present in the ordinary mixed diet. In case 2 on the other hand the cholesterol content of the bile is abnormally low, due probably to extensive liver damage which was indicated by the very marked jaundice. The bile salts were found to be in a concentration of about one-sixth that of normal bile. However, even in this instance and in the following two (cases 3 and 4) in all of which one would expect diminished control of cholesterol metabolism by the liver, diet has no significant effect on the cholesterol concentration of the bile.

A rise in biliary cholesterol concentration occurs in case 5 during the period of high sterol diet, but this cannot be attributed directly to dietary cholesterol as the increase begins before the change of diet takes place.

Fig. VI shows the variations in the volume of bile draining externally and the biliary cholesterol concentration in case 8. The experiment was begun five days after the

operation had been performed. The cholesterol concentration is very low at first, but it gradually rises to a more normal level, the volume of bile draining externally at the same time becoming more constant. The feeding of a large amount of cholesterol, first as the pure substance and then as sheep's brain, produces an immediate increase in the concentration of cholesterol in the bile but this subsequently returns to about the previous level, although the high intake of brain is maintained.

Case 9 is represented in Fig. VII. The experiment was begun one day after the operation. The increased ingestion of cholesterol is, in this instance, undoubtedly accompanied by its increased excretion in the bile.

Finally, Case 10 (shown in Fig. VIII) indicates no increase in concentration of biliary cholesterol on changing to the high sterol diet. In this case also the experiment was begun one day after the operation.

The conclusion reached from the foregoing results is that in general there is no direct correlation between the cholesterol content of the diet and the concentration of cholesterol in the bile collected from biliary fistulae.

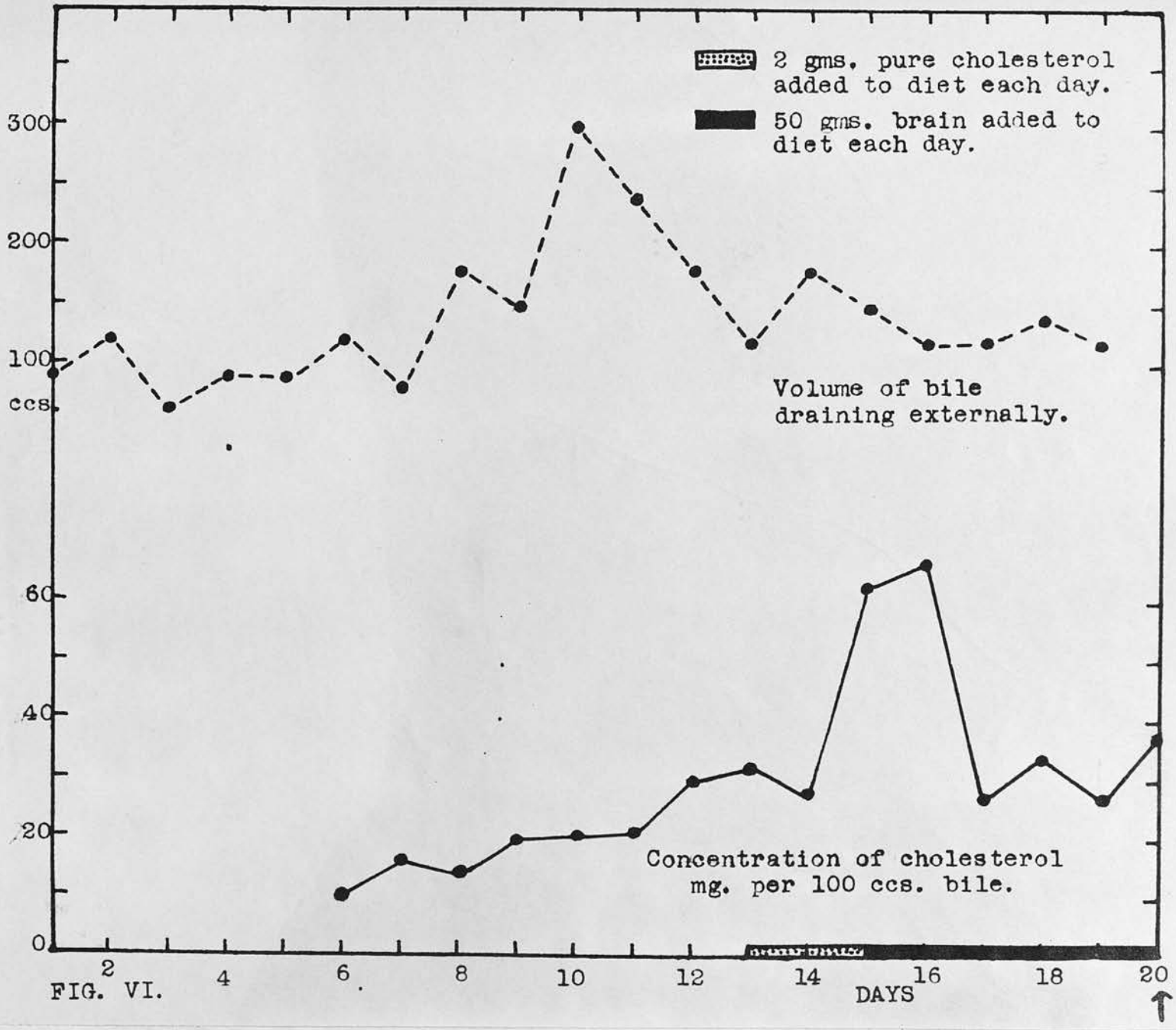

2 gms. pure cholesterol added to diet each day.
50 gms. brain added to diet each day.
300
200
100
ccs.
Volume of bile draining externally.
60
40
20
0
Concentration of cholesterol mg. per 100 ccs. bile.
2
4
6
8
10
12
14
16
18
20
DAYS
Fistula closed.

FIG. VI.

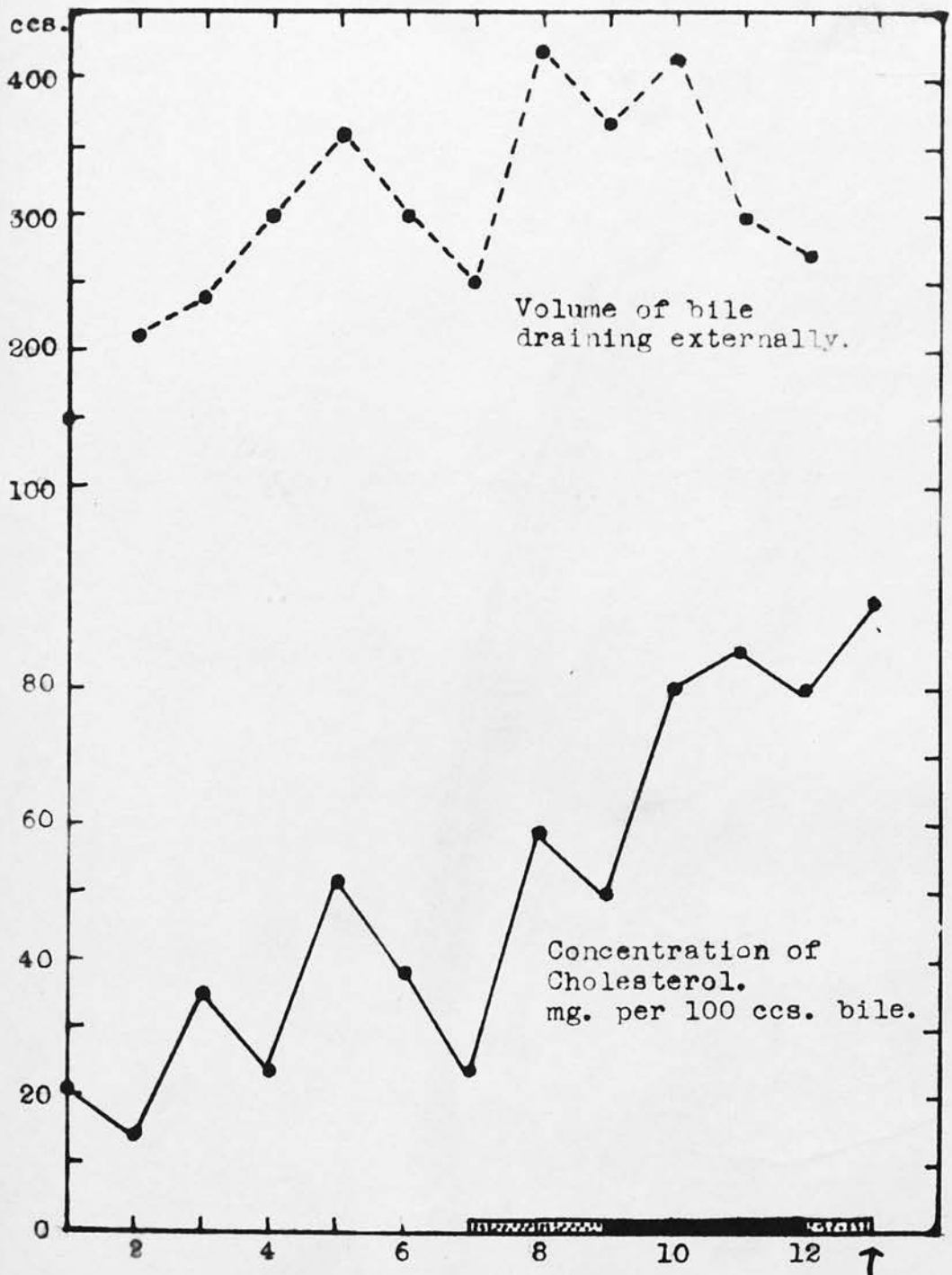


FIG. VII Cholesterol concentration and volume of bile in patient with biliary fistula (minimum of liver damage). Fistula closed.

2 gms. pure cholesterol added to diet each day.

50 gms. brain added to diet each day.

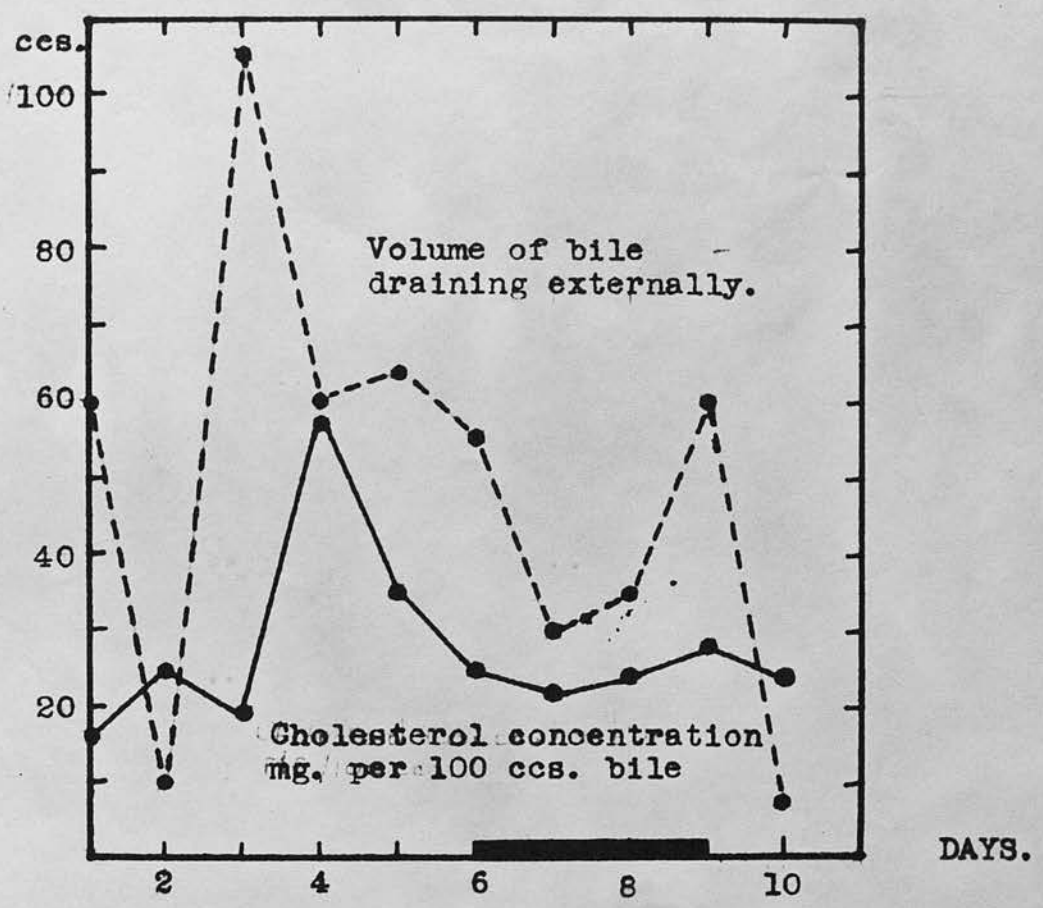


FIG. VIII Cholesterol concentration and volume of bile in patient with biliary fistula.

▬ 50 gms. brain added to diet each day.

4. Effect of a High Sterol Intake on the Output of Cholesterol in the Bile of a Dog with a Total Biliary Fistula.

To overcome the difficulty mentioned above with regard to determining the total output of cholesterol in the bile, recourse must be had to operations on animals in which it can be arranged that all the bile drains externally.

There are several reports on these lines in the literature. Goodman (34) showed that in a dog with a total biliary fistula the quantity of cholesterol found in the bile varied considerably with the nature of the diet but that the actual amount of cholesterol taken in the food was without influence, i.e. white of egg and brain both increase the output to the same extent. This author also states that intravenous injection of cholesterol does not increase the total output in the bile. McMaster (35) used dogs with total biliary fistulae in which the secretion was not exposed to the gall-bladder at any time, and was collected under sterile conditions (method of Rous and McMaster (36)). He observed that immediately after operation the bile was highly pigmented and scanty; then

the volume increased and the bile pigment dropped to a constant level unaffected by diet. The daily cholesterol output appeared to be influenced by dietary changes and rose when large quantities of food were taken. During starvation the bile was scantier and more concentrated, hence the concentration of cholesterol increased, although the total output was less. When a diet rich in cholesterol was given, the total output of cholesterol in the bile greatly increased, possibly due to the fact that a high intake of fat stimulates the secretion of bile. The cholesterol concentration also rose slightly, but this does not seem significant since it rose very much higher during starvation.

Wright and Whipple (37) report that under uniform dietary conditions the "Normal" dog with a biliary fistula eliminates fairly constant amounts of cholesterol (10 - 20 mg. per 100 cc. bile or 0.5 - 1 mg. per kilo per 24 hours). Diets rich in cholesterol (as egg yolk) will raise the cholesterol output in the bile but compared to the dietary intake (1.5 gm. cholesterol) the increase of output in the bile is trivial (5 - 15%). These authors

found that calf's brain was inert, due possibly to lack of cerebrosides and phosphatides.

They also state that bile salt alone will raise the daily cholesterol output in the bile as much as or often more than a cholesterol-rich diet. Bile salt plus egg yolk plus whole bile give maximal figures for excretion of sterol in the bile. This effect again is probably due to increased secretion of bile and not to relative increase of its cholesterol content. On the other hand, Ravdin, Prushankin & Riegel (38) could find no significant change in the daily cholesterol output of the bile on the administration of 2 - 3 gms. sodium dehydrocholate to dogs whose common bile ducts had been intubated.

An increase in the daily biliary excretion of cholesterol in bile fistula dogs after intravenous injection of large amounts of cholesterol has been demonstrated by Fasiani (quoted by Whipple (28)) and D'Amato (also quoted by Whipple) on feeding ox-brain and eggs, in amounts providing about 5 gms. cholesterol per day, to dogs, was able

to increase the sterol output of the bile only from 6 mg. to 8 mg. per day, and suggested that the bile is not the chief avenue of excretion of this substance.

Again, Dostal and Andrews (32) performed experiments on dogs using the Rous two-way fistula and observed no increase in bile cholesterol concentration after the oral administration of either olive oil, butter, cream, meat or bile acids.

Experimental Part.

Professor J. R. Learmouth inserted a tube into the common duct of a dog so that all the bile drained externally (technique according to Markowitz). The animal remained in excellent condition for one month, the absence of bile in the intestine being compensated by giving 1 gm. sodium glycotaurocholate by mouth every day. At no time during this period was the bile re-fed to the dog by stomach tube.

The total volume and the cholesterol concentration of the bile were measured daily. For the first three days insufficient bile was secreted for analysis but thereafter it

increased, although there was considerable fluctuation both in the volume and in the cholesterol content. After about twenty days these became fairly constant (concentration of cholesterol in the bile 13-14 mg. per 100 cc. and daily volume of bile 140-150 ccs., giving a biliary output of about 20 mg. cholesterol per 24 hours).

During this time the dog was maintained on a low-cholesterol diet; for ten days after the operation only milk and dog biscuits were given; subsequently an addition of 50 gms. of lean meat was made to the daily ration. Then, on the 22nd day there was a further addition of 50 gms. ox brain containing about 2 gms. cholesterol, and this diet was maintained for the remainder of the experiment which was unfortunately terminated prematurely by the accidental death of the animal.

As can be seen in Fig. IX, there was an immediate rise in the bile cholesterol (concentration 27 mg. per 100 cc. and total output 38 mg.) as a result of the diet containing brain and this rise persisted for 48 hours, but subsequently both the concentration and total excretion of cholesterol in the bile returned to the original level, although the high sterol content of the diet was maintained.

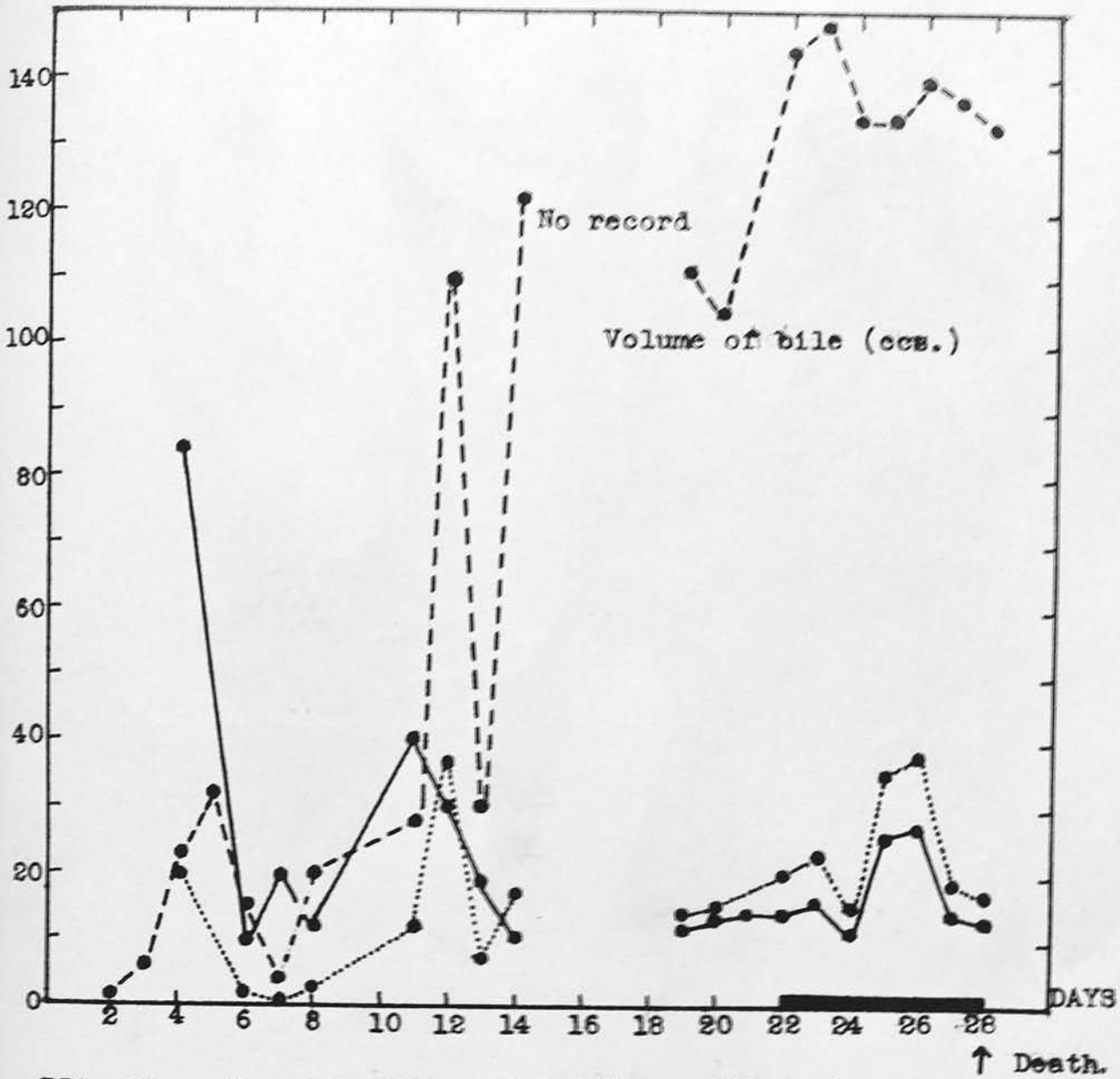


FIG. IX. Concentration and total excretion of cholesterol and volume of bile in dog with total biliary fistula (weight of dog 22 kilos.)

50 gms. brain added to diet each day.

Concentration of cholesterol (mgms. per 100 cc. of bile).

Total excretion of cholesterol (mgms. per 24 hours).

CONCLUSION.

Further experimental work on animals is obviously required before any definite conclusion can be reached as to the effect of diet on the total output of bile and cholesterol, but the evidence of the foregoing experiments on human beings indicates that there is no direct correlation between the intake of cholesterol and its level in the blood and bile. Strong support is given to these observations by a recent (1943) publication by Heymann & Rack (39) who investigated exogeneous cholesterol metabolism in children. These workers were unable to demonstrate any regular response in the serum cholesterol after the administration of single doses of the pure substance dissolved in olive oil and other solvents (cholesterol tolerance curves) or its daily administration for periods of two to four weeks. Furthermore, prolonged administration of "lipoid-free" diets did not lead to low serum cholesterol in their subjects, and they call attention to the confirmation given to their results by observations on children suffering from coeliac disease, in which there is practically no absorption from

the intestine, leading to a state of chronic inanition. In this disease the insufficient absorption of amino acids leads to a definite hypoproteinaemia while the same inability to absorb cholesterol does not invariably decrease the amount of cholesterol in the blood below normal.

Heyman & Rack believe that "the human organism is impressively independent of either the administration or the withdrawal of exogeneous cholesterol", a view which is held by the present writer. Certainly the observations recorded in this paper afford no support to the contention that foods rich in cholesterol should be prohibited in the diet of patients with cholecystitis and cholelithiasis. The exclusion of butter, fat, eggs and liver from the diet of patients with diseases of the biliary tract is based essentially on the fact that gallstones are formed from cholesterol and the hypothesis that a reduction in the intake of cholesterol accordingly reduces the liability to gallstone formation. Such a low-fat, low-cholesterol diet is unphysiological because fat is the natural stimulant to biliary contraction and drainage, and has

the further disadvantage of being unpalatable, monotonous and low in fat-soluble vitamins.

SUPPLEMENT TO PART I

Observations on the Association between Cholesterol and the Plasma Proteins.

The following *in vitro* experiments have no direct connection with the preceding work, but they have some bearing on the solubility of cholesterol in plasma. Cholesterol, both in the free and esterified forms, is water-insoluble and yet it is found "dissolved" in plasma in comparatively high concentration. There must, then, be some constituent of plasma which modifies the properties of cholesterol and enables it to form either a true colloidal solution or a stable chemical complex. In the case of bile, this constituent is usually assumed to be the bile salt fraction and the formation of a water-soluble bile-salt cholesterol complex has been postulated. This explanation, however, does not hold in the case of plasma, since its normal content of bile salt is exceedingly low; indeed some authors claim that bile salt is completely absent.

In certain pathological conditions, for example lipoid nephrosis, the concentration of cholesterol in the plasma is increased almost ten-fold (to about 1,000 mg. per 100 cc),

indicating that plasma is capable of holding in solution amounts of cholesterol far in excess of normal. Yet it is impossible to increase its concentration in normal plasma by shaking plasma with crystalline cholesterol in vitro. Finely-divided cholesterol, obtained by dissolving the crystals in alcohol, throwing out of solution by the addition of water and removing the alcohol by evaporation, was shaken vigorously with plasma of known total cholesterol concentration. The plasma was left to stand overnight, filtered through a fine paper and then re-analysed for total cholesterol by Okey's method. This experiment was carried out several times and in no case was any uptake of cholesterol detected.

It has been suggested by many writers that protein is the constituent of plasma responsible for maintaining cholesterol in solution. Gardner and Gainsborough (37) have summarised the earlier literature and quote from it a considerable weight of evidence in favour of the association of cholesterol with the plasma proteins, particularly the globulin fraction. It is this fraction which is alleged to prevent the extraction of the whole of the cholesterol of serum by simple shaking with

ether, since when the albumin fraction of the serum is high, it is claimed that the amount of cholesterol which can be directly shaken out with ether is high, but when the albumin fraction is low relative to the globulin, the percentage of the cholesterol that can be shaken out with ether is low.

Gardner and Gainsborough publish in their paper the result of one experiment relevant to this globulin-cholesterol association. They separated the various serum proteins by fractional precipitation with ammonium sulphate, and analysed the different fractions for cholesterol. They found that 16.36% of the total sterol was retained with the proteins (13.80% with the euglobulin, 1.03% with the pseudoglobulin and 1.50% with the albumin). 63.3% of the total sterol was found in the filter paper used in filtering the globulin and the remaining 20.4% was lost, possibly in the ammonium sulphate mother-liqours.

These authors state earlier in their paper that colloidal suspensions of cholesterol in saline are readily precipitated by half-saturation with ammonium sulphate, and if the cholesterol in plasma is in a similar state

of colloidal suspension the whole or almost the whole would be precipitated along with the globulin on half-saturation of the plasma with ammonium sulphate. From the result of the above experiment this would appear to be the case; certainly it does not prove the existence of an association between globulin and cholesterol.

A recent paper by McFarlane (41) reports the interesting fact that alternate freezing and thawing of serum enables a high proportion of the total lipoid to be extracted by shaking with ether alone. Without this treatment, negligible amounts of lipoid are extracted into the ether; it is necessary to precipitate the proteins by means of alcohol before complete extraction of the lipoid material can be accomplished. McFarlane suggests that there is a complex formed between globulin and lipoid - the so-called β-globulin - and that this complex is broken down completely by treatment with alcohol, and partially by freezing below -25° C.

Experimental Part.

To investigate this alleged association between plasma proteins and cholesterol, various

methods of precipitating the proteins were tried, and the protein fractions were analysed for cholesterol:

a) The first method of precipitation was that of simple coagulation by heating in a water-bath at 75 - 80° C for about seven minutes. After cooling, the protein gel was washed by stirring throughly with very dilute acetic acid (c.N/1000) and then centrifuged. The supernatant liquid was decanted and the washing and centrifuging were repeated until no chloride could be detected in the supernatant liquid. The protein precipitate was then extracted six times with hot 3:1 alcohol - ether mixture and the extracts made up to 50 ccs., aliquots of which were analysed for total cholesterol.

b) The second method of precipitation was by means of trichloracetic acid. 2 ccs. of serum were placed in a centrifuge tube and the proteins precipitated by the addition of 1 cc. of trichloracetic acid solution (15 gms per 100 ccs.). After centrifuging for 10 minutes, the supernatant liquid

was decanted and the precipitate was washed with very dilute acetic acid. This process was repeated until the washings were free from chloride. Then the precipitate was extracted six times with hot 3:1 alcohol-ether mixture and the extracts made up to 50 ccs.; aliquots of this were analysed for total cholesterol as before.

c) 2 ccs. of serum were precipitated by the addition of 2 ccs. p-toluene sulphonic acid slolution (16 gms. per 100 ccs.) and the procedure was the same as given above (b).

d) 2 ccs. of serum were precipitated with 2 ccs. saturated mercuric nitrate, and again the procedure was the same as outlined above.

The results of these experiments are shown in Table IV.

e) Finally, serum was precipitated with ammonium sulphate so that the proportion of the cholesterol associated with the albumin and globulin fractions could be determined. 2 ccs. of serum

TABLE IV

	Method of Precipitation of Serum Proteins.	Total Cholesterol of Serum. MG/100ccs.	Total Cholesterol of Protein Fraction. MG/1--ccs.	Percentage of Total Cholesterol Precipitated with Proteins.
1.	Heat.	238	206	87
2.	Trichloracetic Acid.	238	221	92
3.	p-Toluene Sulphonic Acid	166	159	95
4.	Mercuric Nitrate.	166	144	87

and 2 ccs. of saturated ammonium sulphate solution were placed in a centrifuge tube and centrifuged. The whole of the supernatant liquid (albumin fraction) was drawn off and 2 ccs. of it were extracted with hot 3:1 alcohol-ether mixture and made up to 50 ccs. Aliquots of this were analysed for free and total cholesterol by Okey's method.

The deposit (globulin fraction) was ground up thoroughly with about 5 ccs. half-saturated ammonium sulphate solution, centrifuged and the supernatant liquid decanted. This process was repeated until the washings showed no turbidity on the addition of trichloracetic acid solution. The deposit was then extracted six times with hot 3:1 alcohol-ether mixture and made up to 50 ccs., aliquots of it being used for estimation of free and total cholesterol.

This whole procedure was carried out twice on each of two different sera. The results are set out in Table V.

TABLE V

	Percentage of Total Cholesterol with Albumin Fraction.	Percentage of Total Cholesterol with Globulin Fraction.	Percentage of Free Cholesterol with Albumin Fraction.	Percentage of Free Cholesterol with Globulin Fraction.	Percentage of Esterified Cholesterol with Albumin Fraction.	Percentage of Esterified Cholesterol with Globulin Fraction.
SERUM A 1	80.7	21.0	75.0	28.9	84.0	16.2
SERUM A 2	84.4	22.5	75.6	21.8	80.4	17.9
SERUM B 1	65.5	24.2	33.1	67.8	66.9	32.2
SERUM B 2	51.6	47.0	45.5	53.0	54.5	44.5

CONCLUSION.

The results set out in Table IV show that a high percentage of the total serum cholesterol is precipitated with the protein fraction in the case of all four methods used for protein precipitation. This may indicate a close association between the cholesterol and protein of serum or it may merely indicate that the cholesterol is in such a form in serum that it is thrown out of "solution" by the methods employed above and hence appears in the same fraction as the proteins. For example, a simple colloidal solution of cholesterol would be precipitated in this way. However, the cholesterol of serum must be different from artificial colloidal solutions of cholesterol, a fact which is obvious from its appearance alone, as was pointed out by Gardner and Gainsborough. The artificial colloidal suspensions are distinctly opalescent, whereas sera, containing much larger concentrations of cholesterol, are often quite transparent. It is possible, however, that serum cholesterol may be in a much higher state of dispersion than the artificial colloidal solutions, which would account for the absence

of the opalescent appearance.

The figures given in Table V are inconsistent and no definite conclusion can be drawn from them, except that they afford no support to the contention that most of the cholesterol of serum is precipitated with the globulin fraction of the proteins. They indicate, if anything, that the greater part of the cholesterol remains with the albumin fraction, a fact which per se points to some association between the cholesterol and serum protein, since a simple colloidal solution of cholesterol would be precipitated by half-saturation with ammonium sulphate. These figures show no significant difference in behaviour between the free and esterified forms of cholesterol.

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ACKNOWLEDGEMENTS.

My thanks are due to Professor L. S. P. Davidson and to Dr. C. P. Stewart, under whose direction this work was done, and to Professor J. R. Learmouth, who carried out the necessary animal surgery.

Part of the expenses were defrayed by a grant from the Medical Research Council to Professor Davidson.

PART II

STUDIES ON THE OXIDATION OF COLLOIDAL AQUEOUS SOLUTIONS OF CAROTENE.

PART II

Introduction.

The unsaturated hydrocarbons, α-, β- and γ-carotene readily undergo autoxidation, particularly under the influence of light. No information is available on the products of oxidation by molecular oxygen, but it has been established that oxidation of β-carotene by means of potassium permanganate gives a mixture of acetic acid, 1:1-dimethyl glutaric acid, 1:1-dimethyl succinic acid and dimethyl malonic acid, and that oxidation by means of ozone gives geronic acid. The oxidation products are colourless so that the progress of oxidation can be followed either by the loss of colour or by the oxygen uptake of the carotene solution.

Most of the work on the oxidation of the carotenes has been carried out on solutions of the hydrocarbons in organic solvents. Baur (1) and (2) used α-carotene dissolved either in chloroform, cyclohexane or octane and measured the oxygen uptake at various oxygen pressures on exposure to the light from a mercury vapour lamp. Over the period of the experiment the solutions were kept in a cold-water thermostat at 13° C. The concentration of carotene used

was high - 50 mg. in 25 ccs. of solvent. From the results of his experiments, Baur considers that the initial reaction is a photo-oxidation, involving the formation of unstable photo-oxides and that subsequently these photo-oxides dissociate and oxidation proceeds, independently of the presence of light, with splitting of the carbon chain and consequent bleaching of the carotene. He found that, in the dark, carotene absorbs oxygen only after a long period of time (at 13°C.).

Chevallier, Matheron and Roux (3) measured the oxygen uptake (by Warburg's manometric method) of solutions of vitamin A in alcohol and found that, for the free vitamin, the oxygen consumption is very rapid initially and then decreases progressively. If the vitamin is in the esterified form, there is a considerable lag period and then the consumption of oxygen accelerates gradually. These authors give no figures in support of their statements.

There is little information on the oxidation of carotene in colloidal aqueous solution. The only extensive investigations were carried out by Karrer and Strauss (4). These workers prepared colloidal solutions by dissolving the pigment in acetone, pouring the resulting solution into aqueous sodium cholate solution

and then removing the acetone by distillation in vacuo. By this means they obtained two varieties of colloidal solution, one a very concentrated "red solution" (low dispersion of the colloid) and the other a "yellow solution", approximately twenty times more dilute (high dispersion of the colloid). The size of the particles appeared to affect the rate of oxidation of the carotene, the red solutions being considerably more sensitive to light and heat than the yellow solutions.

Karrer followed the progress of oxidation by measurement of the loss of colour in the colloidal solutions by means of the absorption spectrograph. At 20° C in sunlight colloidal solutions of β-carotene (500 γ per 100 ccs.) were bleached in about 4 hours, whereas in the dark no bleaching was apparent during that time. Bleaching was inhibited by addition of ascorbic acid or lecithin to the colloidal carotene solutions and accelerated by addition of dl-alanine or gelatine.

Karrer and Strauss then investigated the effect of albumin and lecithin on colloidal solutions of carotene. They state that, in the presence of albumin, carotene takes on the properties of a chromoprotein in place of those

of a lipochrome. It becomes soluble in water from which it cannot be extracted by fat solvents, and it is precipitated with the proteins by protein precipitants.

Lecithin, also, has a modifying effect on colloidal carotene. Karrer and Strauss prepared lecithin-containing solutions of colloidal β-carotene in two ways. In the first case, a colloidal aqueous solution of lecithin was added to the colloidal carotene solution, and it was found that the absorption spectrum of the carotene in the resulting mixture remained unchanged. In the second case, lecithin was dissolved in an alcoholic solution of the carotene, the solution thrown into water and the alcohol distilled off *in vacuo*. The absorption spectrum of the carotene changed, indicating the formation of some complex between the carotene and lecithin.

Colloidal solutions containing carotene, lecithin and albumin were also prepared, and it was found that the carotene again took on the properties of a chromoprotein. The pigment was precipitated with the proteins by ammonium sulphate and it could not be extracted from them by chloroform or benzene. Karrer and Strauss believe that colloidal aqueous carotene solutions

containing protein and lipoid closely resemble serum carotenoids. In both cases their light-sensitivity is very small in contrast to that of colloidal carotene solutions containing only protein; in both cases the carotene is precipitated by ammonium sulphate; and in both cases no trace of pigment is extracted by organic solvents.

Several papers have been published on the subject of the alleged "carotene oxidase", an enzyme present in soya beans which was claimed by Bohn and Haas (5) to have an accelerating effect on the oxidation of carotene. This claim was supported by Sumner and Dounce (6). It was, however, found by Tauber (7) and later by Strain (8) that "carotene oxidase" as described by the authors does not exist, and that the oxidation of carotene is caused indirectly by an unsaturated-fat oxidase present in soya bean meal. The carotene used by the earlier workers was dissolved in vegetable oils, so that the unsaturated fats contained in those oils would be oxidised by the action of the enzyme and the intermediate products of fat oxidation would in turn accelerate the oxidation of the carotene. This effect was not observed if the carotene was dissolved in mineral oil. The same conclusion

was reached by Sumner and Sumner (9)

Experimental.

The following investigations were undertaken with the object of studying the effect of some of the constituents of blood on the oxidation of colloidal carotene solutions in the hope of throwing some light on the mechanism of preservation of carotene in that fluid. An artificial colloidal solution of carotene is bleached fairly rapidly (in about 24 hours) at 37° C, even in the dark, whereas the carotene of serum will remain unchanged under the same conditions for very much longer periods of time (several days). The following figures illustrate this property of the carotene in different sera incubated at 37°C in the dark (Table I). Methods of extraction and estimation of the carotene will be described later.

Serum carotene differs completely from that in artificial solution, or else its colloidal state is profoundly modified by one or more of the constituents of serum. The observations of Karrer (described above) suggest some association between the carotene, proteins and lecithin of serum. The solubility of the carotene is altered in the presence of protein and it can no longer be extracted by fat solvents, facts which alone indicate some change in state.

TABLE I

HOURS	SERUM A	SERUM B	SERUM C	SERUM D	CAROTENE SOLUTION	CAROTENE SOLUTION
	γ per 10cc. serum	γ per 10cc. serum	γ per 10cc. serum	γ per 10cc. serum	γ per 5 cc	γ per 5cc.
0	12.5	10.8	18.2	9.0	15.5	25.0
24	12.5	10.5	17.5	8.7	nil	2.2
48	13.7	11.5	17.0	8.0	-	-
72	11.2	-	13.5	-	-	-
96	-	10.0	-	7.2	-	-
120	8.9	9.5	8.9	-	-	-

(The precipitation of the pigment with the proteins by means of ammonium sulphate has no significance since ammonium sulphate will precipitate carotene from an artificial colloidal solution, although the precipitation is complete only after 24 hours). It is possible, then, that the serum proteins and lecithin are wholly or partially responsible for delaying the oxidation of serum carotene.

METHODS.

Since it is not possible at the present time to obtain pure β -carotene, mixed carotene (B.D.H.) was used in all the experiments. This preparation is mainly β -carotene with a small proportion of the α and γ varieties (approximately 90% β -carotene).

Storing of the Carotene Powder.

Some difficulty in keeping the powder was encountered at first, since it readily undergoes oxidation on exposure to air. The first method of preservation tried was to dissolve in petroleum ether and keep in an atmosphere of nitrogen. Bleaching was prevented, but some unknown change took place so that the carotene was no longer capable of being bleached even in colloidal solution. Colloidal solutions prepared from it showed no bleaching over periods of several days and no uptake of oxygen in the Warburgmanometric apparatus. Hence, this method of preservation had to be rejected. Neither sealing the container after use nor keeping in a desiccator over alkaline pyrogallol completely prevented oxidation, but finally a solution of the problem was found by keeping the carotene powder in a long glass tube and sealing

in vacuo. The tube was re-sealed in vacuo each time after use, and by this means the carotene was kept unchanged for as long as it was required.

Preparation of the Colloidal Solutions.

Colloidal solutions were prepared by dissolving varying amounts of the carotene powder in 10 ccs. of acetone and pouring the solution into about 200 ccs. distilled water (distilled in an ordinary metal still). The acetone was subsequently removed by distillation in vacuo. During the distillation, in order to minimise the oxidation of the carotene, the temperature of the water-bath was maintained below 45°C, the solution was shielded from the light, and the capillary inlet was connected with a nitrogen cylinder. Complete removal of the acetone was accomplished by this method (nitroprusside test negative). Prolonged exposures to paraffin wax shavings or to sodium bisulphite solution in a vacuum desiccator were unsatisfactory as methods of removing the acetone.

Estimation of the Carotene.

5 ccs. samples of the colloidal solution were extracted into petroleum ether (b.p. 40-60°)

by shaking in a separating funnel. It was necessary to add absolute alcohol to at least 40% by volume before complete extraction of the carotene could be accomplished. (The alcohol used was aldehyde-free, purified according to Dunlap's method (13)). The samples were extracted twice with 4 ccs. of petroleum ether and the extracts made up to 10 ccs. The yellow colours of these extracts were compared in a Spekker Photoelectric absorptiometer, using the blue filter No. 6 (absorption maximum 480 mμ.) A specially constructed standard curve was used to translate the extinction coefficients into terms of concentrations of carotene. This curve was constructed with pure β-carotene, obtained at an earlier date, hence all results are expressed in terms of β-carotene, although the carotene actually used for the experiments contained small amounts of α and γ-carotene.

Experimental Conditions.

Tubes containing the colloidal carotene solutions were suspended in a thermostat at 37°C. and air was drawn slowly through the series of tubes to ensure that each

tube received a sufficient supply of oxygen. It was thought that bubbling pure oxygen through the tubes might be more favourable to the oxidation than air, and so a comparison was made between the two, both at room temperature and at 37°C. (See Table II)

It will be seen from the table that there is a slight increase in the rate of bleaching when oxygen is bubbled through the solution instead of air, but the difference was not sufficiently great to justify the additional expense of using oxygen.

All experiments were carried out in the absence of sunlight, the only light being that given by the bulbs used for heating the thermostat. Since serum carotene is normally in the dark, it was considered essential that any comparison between that and artificial colloidal carotene should be made in the absence of sunlight.

Comparison of Oxygen-Uptake and Bleaching of Carotene.

To establish the relationship between the bleaching of carotene solutions and their uptake of oxygen, the Warburg Manometric technique (direct method) was used. 2 ccs.

TABLE II

	AIR		OXYGEN	
HOURS	20°C. γ per 5cc. Colloidal Solution.	37°C. γ per 5cc. Colloidal Solution	20°C. γ per 5cc. Colloidal Solution	37°C. γ per 5cc. Colloidal Solution
0	13.0	13.0	13.0	13.0
2	13.5	12.0	12.5	11.0
4	13.0	10.5	13.0	8.0
6	12.8	8.5	12.8	7.0

Progressive bleaching of colloidal carotene solution over a period of six hours.

of colloidal carotene solution and 1 cc. of phosphate buffer (pH 7.2) were added to each flask. A roll of starch-free filter-paper (Whatman No. 40) soaked with 0.3 ccs. 20% KOH was placed in the inner compartment to ensure the absorption of any carbon dioxide which might possibly be formed. The temperature of the thermostat was 37°C. and the rate of shaking about 100 oscillations per minute. After equilibration (10 mins) the oxygen uptake of the carotene was measured over a period of about 10 hours, readings being taken at varying intervals. (It was not possible to prolong these experiments further as the solutions were not sterile and the results would have been valueless owing to bacterial respiration).

Simultaneously, the bleaching of the same preparation of colloidal carotene solution was measured colorimetrically as described above. The tube containing the solution was aerated in the thermostat and 5 cc samples, from which petrol ether extracts were made, were taken every hour.

The progress of the bleaching and the oxygen consumption are compared in Figs. I and II. There is no close correspondence

between the bleaching and oxygen-uptake curves, but this discrepancy can be explained by the fact that mere loss of colour does not mark the cessation of oxidation; the colourless oxidation products themselves undergo oxidation and continue to absorb oxygen even though the bleaching has almost come to a standstill. Since the colorimetric technique is simpler than the manometric one, and allows addition of the ~~the~~ test solutions of other substances liable to absorb oxygen (e.g. ascorbic acid) and also of serum itself, without the attendant difficulties connected with the use of these in the Warburg method, it was used in all subsequent experiments.

The effect of temperature, also, can be seen from Figs. I and II. The bleaching of the carotene at room temperature (20°C.) is much slower than the bleaching of that at 37°C.

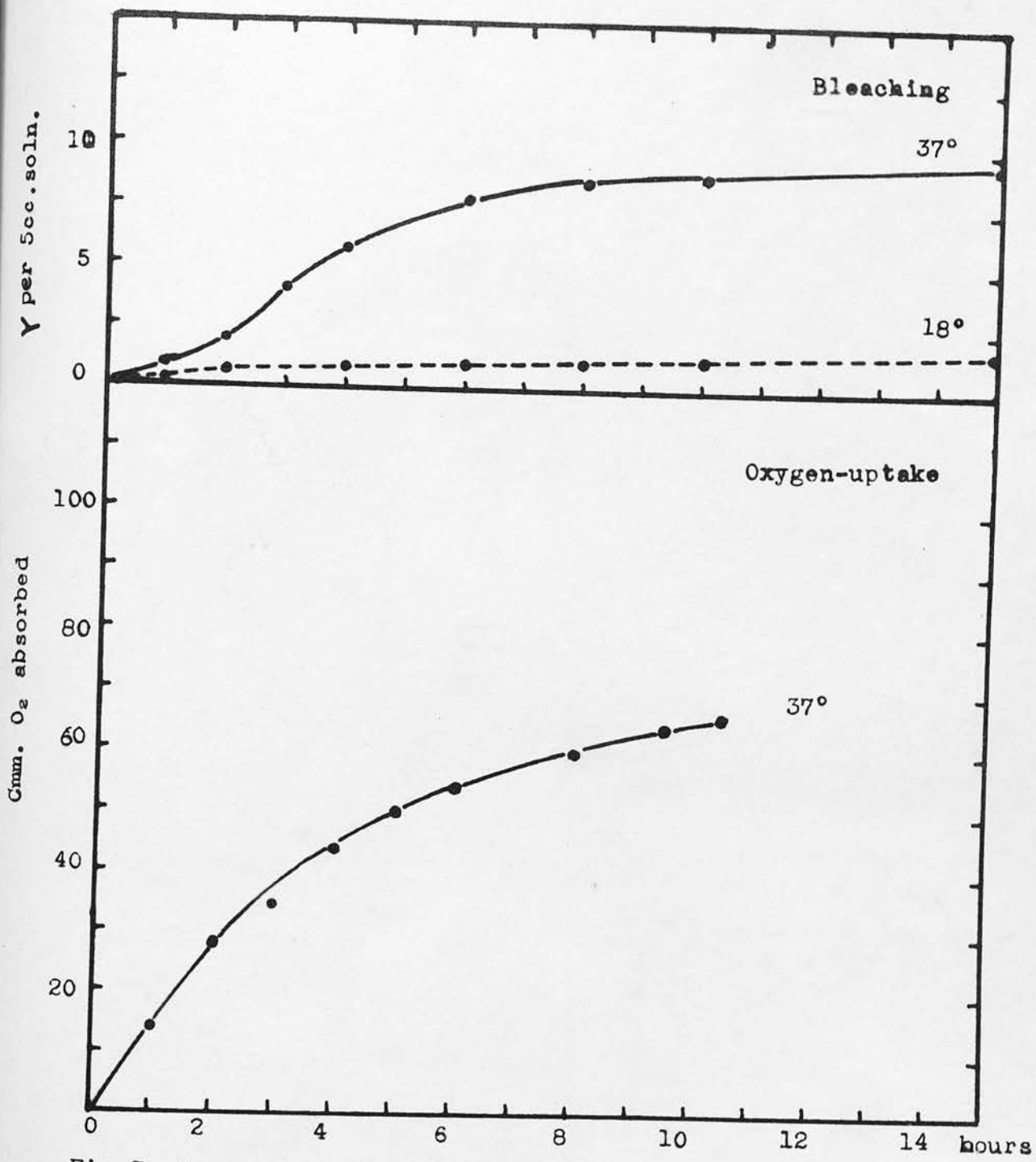

Fig.I Comparison of oxygen-uptake and bleaching of carotene

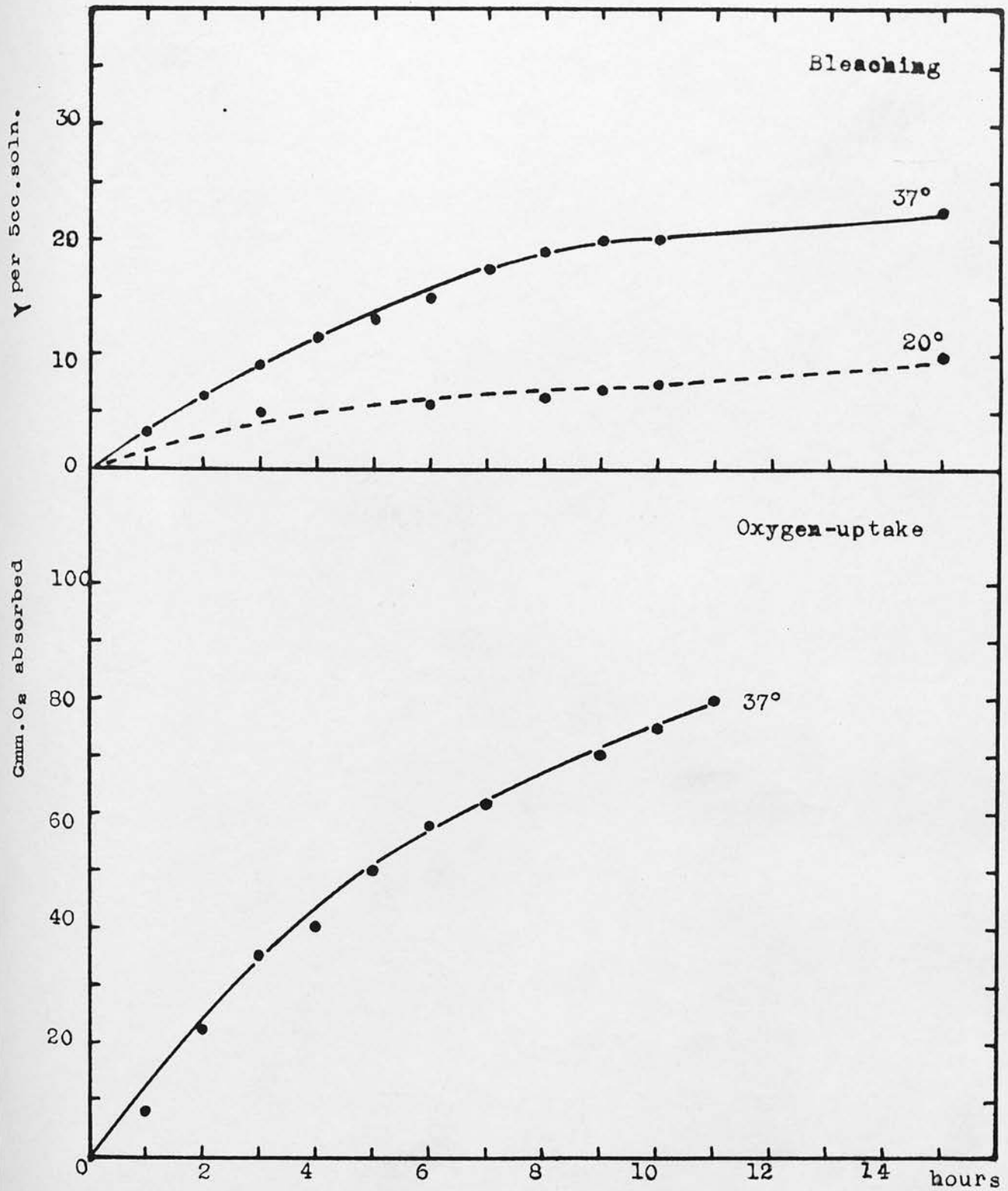

Fig.II Comparison of oxygen-uptake and bleaching of carotene

EXPERIMENTAL RESULTS AND DISCUSSION.

a) Effect of pH on the Oxidation of Carotene.

Karrer makes no mention of the effect of pH on colloidal carotene solutions. It is possible that small changes in pH may influence the rate of oxidation of carotene, hence specimens of a colloidal carotene solution, prepared as described above, were buffered at varying pH values by the addition of phosphate buffers (mixtures of M/150 Na_2HPO_4 ~~Na_2HPO_4~~ and M/150 KH_2PO_4). 15 ccs. of carotene solution and 10 ccs. of buffer mixture were placed in each tube. The pH of each solution was checked at the end of the experiment to ensure that the buffering power was adequate. Since it was possible that the phosphate per se might have some effect on the oxidation, a control tube containing distilled water in place of the buffer mixture was set up. Samples from each tube were removed at varying intervals (see Tables III - VII) and the carotene extracted by petroleum ether and estimated colorimetrically. The results are shown in Tables III - VII.

TABLE III

HOURS	TUBE A no buffer. pH 5.9	TUBE B pH 5.8	TUBE C pH 7.1	TUBE D pH 8.2
	γ per 5ccs. solution	γ per 5 ccs. solution	γ per 5 ccs. solution.	γ per 5 ccs. solution.
0	8.8	8.8	8.8	8.8
0.5	6.3	6.3	5.8	5.8
1.25	4.0	4.0	4.2	3.8
2	2.8	2.5	2.5	2.5
3	1.5	0.8	0.8	0.7
4.5	0.7	0.0	0.0	0.0

TABLE IV

HOURS	TUBE A no buffer pH 6.8	TUBE B pH 6.1	TUBE C pH 7.1	TUBE D pH 7.7	TUBE E pH 8.3
	γ per 5 ccs. solution	γ per 5 ccs. solution	γ per 5 ccs. solution	γ per 5 ccs. solution	γ per 5 ccs. solution
0	14.0	14.0	14.0	14.0	14.0
1	9.5	9.5	9.8	9.5	9.5
2	7.3	7.3	6.8	4.5	6.5
6	4.0	4.2	3.8	1.3	3.5
8.5	3.0	3.5	3.3	1.0	2.5
20	0.8	1.0	1.1	0.8	1.2

TABLE V

HOURS	TUBE A no buffer pH 5.3	TUBE B pH 5.9	TUBE C pH 6.5	TUBE D pH 7.0	TUBE E pH 8.0
	γ per 5 ccs. solution	γ per 5 ccs. solution	γ per 5 ccs. solution	γ per 5 ccs. solution	γ per 5 ccs. solution
0	34.2	34.2	34.2	34.2	34.2
1.5	24.1	24.9	22.4	24.1	23.6
3.5	15.5	15.5	16.2	16.2	16.4
5.5	14.7	15.8	15.0	15.5	15.0
18	9.5	10.3	10.3	10.3	8.9

TABLE VI

HOURS	TUBE A no buffer pH 5.7	TUBE B pH 5.8	TUBE C pH 6.5	TUBE D pH 6.9	TUBE E pH 7.8
	γ PER 5 ccs solution	γ per 5 ccs. solution	γ per 5 ccs. solution	γ per 5 ccs. solution	γ per 5 ccs. solution
0	34.5	34.5	34.5	34.5	34.5
3	24.3	24.3	24.0	25.5	23.5
8	12.4	12.8	11.8	12.8	12.5
20	9.3	9.9	8.6	8.9	8.6

TABLE VII

HOURS	TUBE A no buffer pH 6.7	TUBE B pH 5.8	TUBE C pH 6.4	TUBE D pH 6.9	TUBE E pH 7.8
	γ per 5 ccs. solution	γ per 5 ccs. solution	γ per 5 ccs. solution	γ per 5 ccs. solution	γ per 5 ccs. solution
0	51.5	51.5	51.5	51.5	51.5
4	30.8	33.0	33.7	33.5	32.8
8	18.2	17.7	18.2	16.8	18.0
20	12.5	12.8	11.9	12.1	12.6

The figures given in the foregoing tables show no significant differences between the rates of oxidation of carotene at pH values varying between 5 and 8, and hence no variation in the pH of serum is likely to affect the serum carotene. Certainly, the figures for Tube D in Table IV show apparent slight acceleration of oxidation, but since this is inconsistent with the results of the other experiments, it must be a technical error.

The rate of oxidation of carotene, then, is independent of small changes of pH within the range investigated. High acidity (pH 1-3) causes precipitation of the colloid round the sides of the tube and hence apparent acceleration of the oxidation in the samples taken for estimation of the pigment.

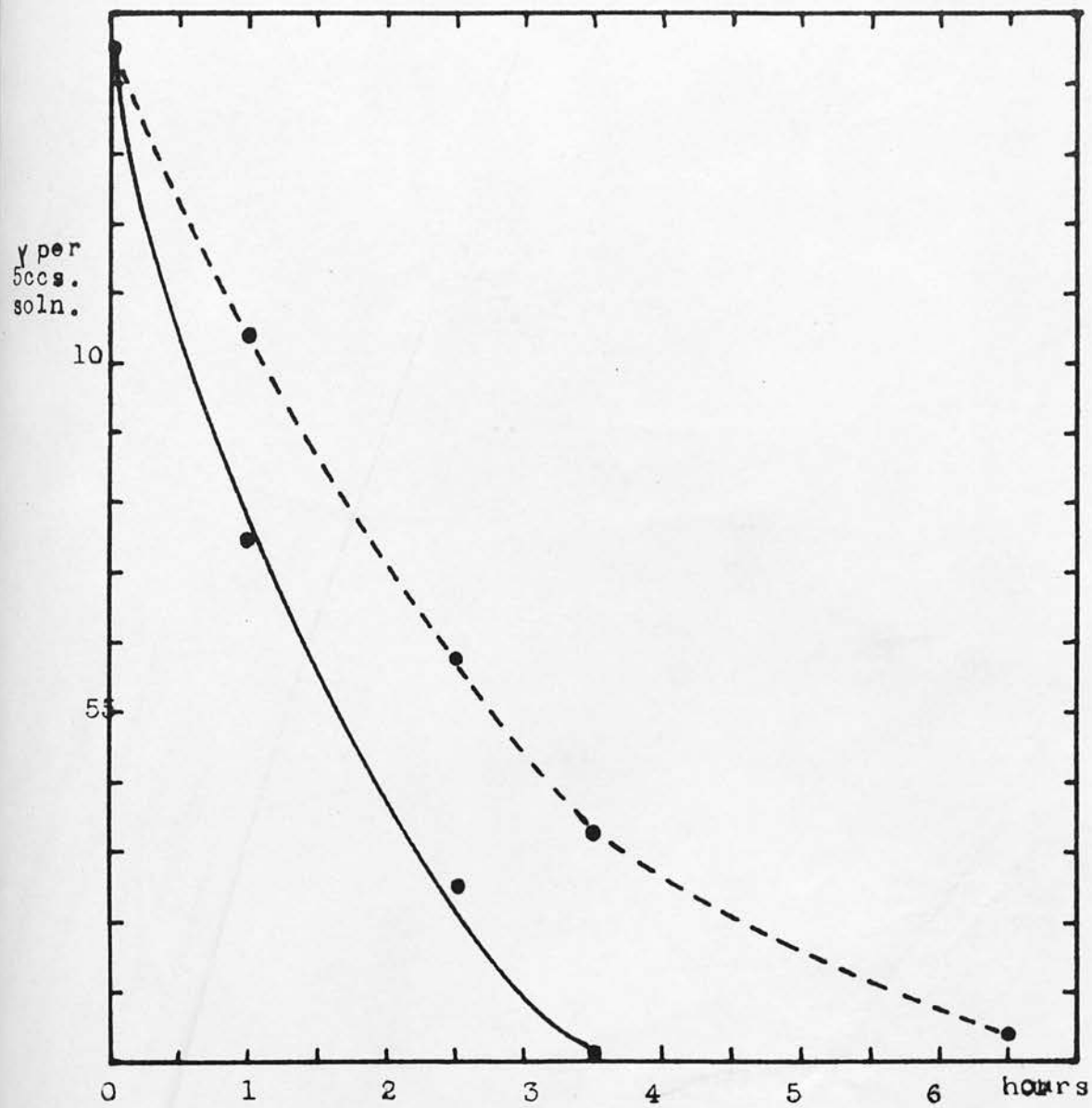

Fig.III. The effect of ascorbic acid on the rate of oxidation of carotene in colloidal solution.

——Control
---10 mg. ascorbic acid added to the carotene soln.

b) Effect of Ascorbic Acid on the Oxidation of Colloidal Carotene.

Karrer (4) states that addition of ascorbic acid to colloidal solutions of carotene inhibits the oxidation of the pigment. An experiment was carried out to verify this and the results are represented in Fig. III. 10 mg. of ascorbic acid were added to one tube (see Fig. III) giving an initial concentration of 33 mg. ascorbic acid per 100 cc. of solution. At the beginning of the experiment the molecular proportion of ascorbic acid to carotene was 346: 1. From the figure it will be seen that there is quite significant inhibition of oxidation of carotene in the presence of ascorbic acid, due, no doubt, to the reducing properties of that substance. If that is the case, one would expect that other substances having reducing properties would also inhibit the oxidation of carotene. An experiment was carried out to compare the inhibiting powers of ascorbic acid, glutathione and glucose. Table VIII shows the results.

At the beginning of the experiment the molecular proportions were ascorbic acid 364: glutathione 209: glucose 356: carotene 1. The

TABLE VIII

HOURS	TUBE A	TUBE B Ascorbic Acid.	TUBE C Glutathione.	TUBE D Glucose.
	γ per 5 ccs. COLLOIDAL SOLUTION	γ per 5 ccs. COLLOIDAL SOLUTION	γ per 5 ccs. COLLOIDAL SOLUTION	γ per 5 ccs. COLLOIDAL SOLUTION
0	13.8	13.8	13.8	13.8
1	6.8	6.6	7.3	6.8
2	3.5	4.3	4.4	3.8
3	2.3	3.8	3.7	2.5
4	0.5	3.0	2.8	0.6

Tube A contained 30 ccs. carotene solution. (CONTROL).
Tube B " " " " " plus 10 mg. ascorbic acid.
Tube C " " " " " plus 10 mg. glutathione.
Tube D " " " " " plus 10 mg. glucose.

ascorbic acid and glutathione show slight inhibiting power, whereas glucose is without effect.

Since it was probable that the ascorbic acid and the glutathione would themselves be oxidised during the aeration of the solutions, and hence lose their power of inhibiting the oxidation of carotene, the experiment was repeated using larger amounts of the reducing substances initially and adding a second quantity in the course of the experiment (see Table IX).

In this case the initial molecular proportions were ascorbic acid 2415: glutathione 1384: glucose 2361: carotene 1. Again it is apparent that there is a slight but significant inhibition of oxidation by ascorbic acid and by glutathione, whereas glucose is ineffective. It is improbable, however, that either ascorbic acid or glutathione is responsible for preserving serum carotene from oxidation since the proportion of these substances used in the foregoing experiment are far in excess of those found in serum and yet their inhibiting power is relatively feeble.

TABLE IX

HOURS	TUBE A	TUBE B Ascorbic Acid.	TUBE C Glutathione.	TUBE D Glucose.
	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5 ccs. colloidal solution
0	21.0	21.0	21.0	21.0
1	15.0	17.3	17.3	15.3
2	14.5	17.2	17.8	13,9
4	14.0	17.0	16.8	13.7
→ 6.5	12.5	16.8	15.5	12.2
23	9.5	14.0	13.3	10.5

Tube A contained 30 ccs. carotene solution (CONTROL).
Tube B " " " " " plus 100 mg. ascorbic acid.
Tube C " " " " " plus 100 mg. glutathione.
Tube D " " " " " plus 100 mg. glucose.

A further 100 mg. of the appropriate reducing substance was added to Tubes B,C and D $6\frac{1}{2}$ hours after the experiment had begun.

c) Effect of Unsaturated Fats on the Rate of Oxidation of Colloidal Carotene Solutions.

Tauber (7) and Strain (8) and others, in the course of their investigations on the alleged "carotene oxidase", found that the intermediate oxidation products of the unsaturated fatty acids linoleic, linolenic and ricinoleic had an accelerating effect on the oxidation of carotene. Their experiments were carried out on carotene dissolved in fat solvents, and so it was decided to test this effect in the case of colloidal carotene.

The effect of unoxidised unsaturated fats was tried in the first instance. A suspension was prepared by shaking 2 ccs. linseed oil with about 20 ccs. distilled water for a few minutes and then filtering. The amount of oil in the filtrate was very small and it was found to have no influence on the bleaching of carotene in colloidal solution. The experiment was repeated the following day and it was found that there was a marked acceleration of bleaching in the tube containing the suspension. This effect was considered to be due to the partial oxidation of the unsaturated fats in the

suspension and a comparison was made between the effect of a freshly-prepared suspension of linseed oil and that of a suspension prepared in the same way but allowed to stand overnight. The experiment was performed on two different preparations of colloidal carotene and the results are shown in Fig. IV. Both sets of curves (A and B) indicate that the old linseed oil suspensions have a marked accelerating effect on the oxidation of carotene, and that this property is not shown by the freshly-prepared suspensions.

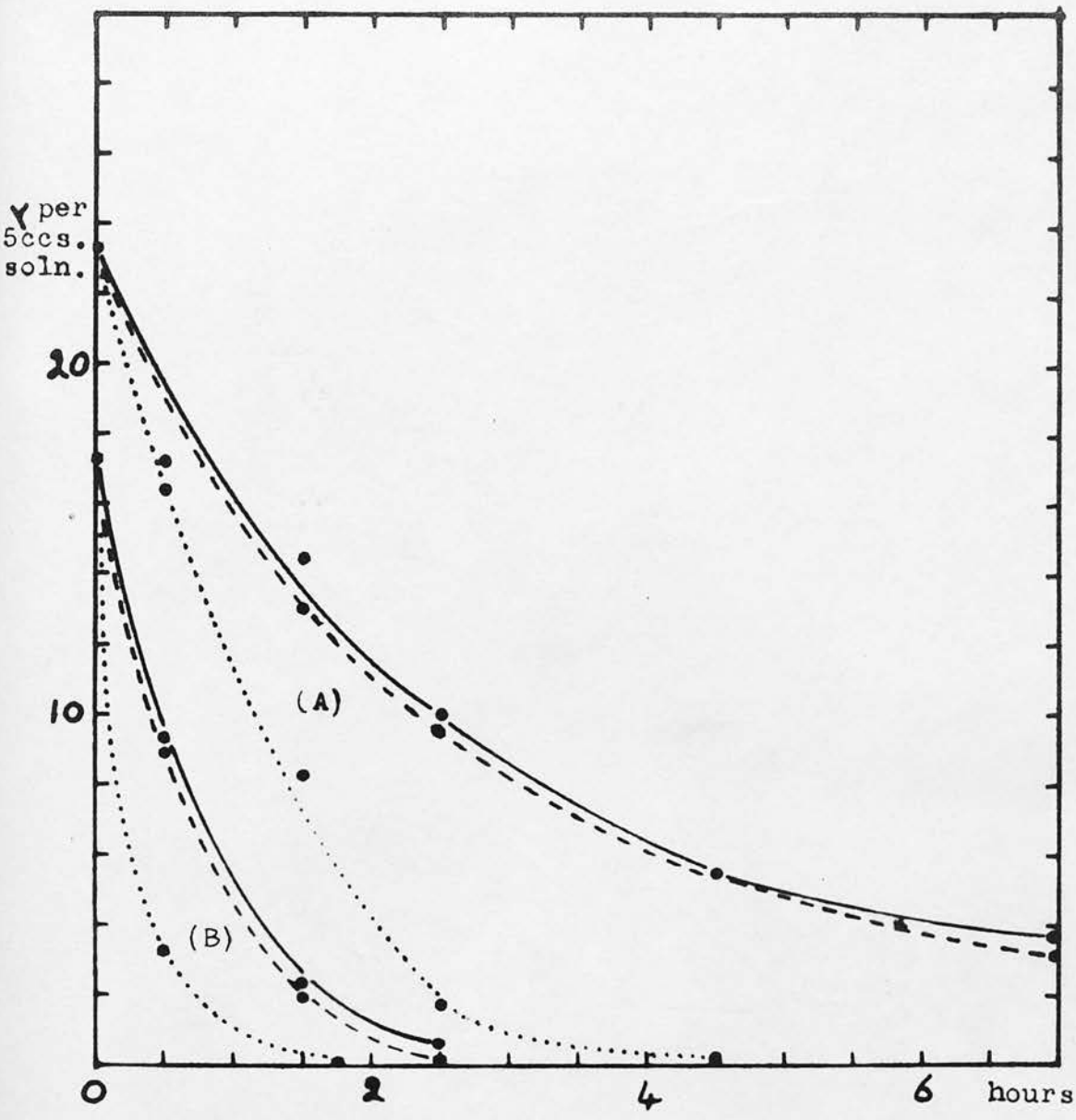

Fig.IV. The effect of oxidised unsaturated fats on the oxidation of carotene in colloidal solution.

—— Control
– – – Solution containing fresh oil suspension
····· Solution containing old oil suspension

d) Effect of Bile Salts on the Rate of Oxidation of Colloidal Carotene

Karrer states in his paper that sodium cholate has an inhibiting effect on the oxidation of the carotenes, but that this effect is far more marked in the case of α-carotene than it is in the case of β-carotene. It is possible that bile salts in serum may play some part in the preservation of serum carotene.

There are conflicting reports of the amounts of bile salt in human serum. Gregory and Pascoe (10), using a colorimetric method, found none at all, although most authors claim to have found small amounts (amino-nitrogen method). The figure 10 mg. per 100 cc. was taken as the average estimate (from Harrison's "Chemical Methods in Clinical Medicine").

A colloidal solution containing approximately five times the amount of carotene usually present in human serum was prepared. Sodium glycocholate (Hoffmann - La Roche) was added to tubes containing the colloidal solution so that the proportion of bile salt to carotene corresponded roughly (a) to that

found in serum and (b) to five times that found in serum. The tubes were subsequently aerated in the dark at 37°C in the usual way. To a third sample of colloidal solution sodium tauroglycocholate (B.D.H.) was added so that the proportion of bile salt to carotene corresponded approximately to that normally found in serum. The rate of oxidation of carotene in these three tubes was compared with that in the control tube (no bile salt added). Table X shows the results of two such experiments.

It will be seen from Table X that neither sodium glycocholate nor sodium tauroglycocholate has a stabilising effect on carotene in concentrations corresponding to, or five-fold that which is found in serum. It is, then, apparent that bile salt can take no part in the preservation of serum carotene, but further experiments were carried out to test the effect of greatly increased concentrations of bile salts and the results are set out in Tables XI and XII.

TABLE X

	HOURS	TUBE A γ per 5 ccs. colloidal solution	TUBE B γ per 5 ccs. colloidal solution	TUBE C γ per 5 ccs. colloidal solution	TUBE D γ per 5 ccs. colloidal solution
Expt. I	0	25.0	25.0	25.0	25.0
	1	15.5	15.8	15.8	15.1
	2.5	9.0	9.0	9.5	8.8
	5	6.3	5.5	6.2	6.0
	22	2.0	1.3	1.5	1.0
Expt. II	0	25.6	25.6	25.6	25.6
	1	13.9	15.2	14.8	14.5
	3	7.5	7.0	7.2	6.8
	22	0.0	1.2	0.0	0.8

Tube A contained 20 ccs. colloidal carotene solution (CONTROL)
Tube B " " " " " plus 10 mg. sod. glycocholate. (proportion corresponded to that in serum)
Tube C " " " " " plus 50 mg. sod. glycocholate (proportion corresponded to 5 times that in serum)
Tube D " " " " " plus 10 mg. sod. tauroglycochol. (proportion corresponded to that in serum)

TABLE XI

HOURS	TUBE A	TUBE B	TUBE C	TUBE D	TUBE E
	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution
0	25.0	25.0	25.0	25.0	25.0
1	15.6	16.4	16.2	16.2	17.5
2	10.0	9.2	9.2	9.5	12.2
3	6.2	6.2	5.7	5.9	7.9
6	2.5	2.8	2.5	2.3	5.0
24	0.0	0.0	0.0	0.0	1.5

		Bile salt/Carotene
Tube A contained 30 ccs. carotene solution	(CONTROL).	
Tube B " " " " "	plus 5 mg. sod. glycocholate	33.3
Tube C " " " " "	plus 10 mg. " "	66.7
Tube D " " " " "	plus 20 mg. " "	133.3
Tube E " " " " "	plus 25 mg. " "	166.7

TABLE XII

HOURS	TUBE A	TUBE B	TUBE C	TUBE D	TUBE E
	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution
0	22.3	22.3	22.3	22.3	22.3
1	11.9	20.0	21.2	21.2	21.9
2	10.0	16.2	17.5	18.7	19.3
4	2.5	11.2	12.5	15.6	16.9
6	0.0	8.7	10.5	13.7	16.2
21	-	1.5	1.5	2.7	5.6

					Bile Salt/ Carotene
Tube A contained	30ccs.	carotene	solution	(CONTROL).	
Tube B	"	"	"	plus 50 mg. sod. glycocholate	372
Tube C	"	"	"	plus 100 mg. " "	744
Tube D	"	"	"	plus 200 mg. " "	1488
Tube E	"	"	"	plus 500 mg. " "	3720

Combining the results of these two tables, it is apparent that an extremely high proportion of bile salt to carotene is required before the bile salt exerts any inhibitory action on the oxidation of carotene. When, however, the ratio has reached a sufficiently high value, increasing concentration of bile salt results in increasing inhibition of oxidation of carotene.

Thus:-

Bile salt / Carotene.	Percentage inhibition of Oxidation in 6 hours.
33.3	0
66.7	0
133.3	0
166.7	6
372	39
744	47
1488	62
3720	73

Fig. V represents these figures graphically.

It was noticed in the course of these latter experiments that increasing

concentration of sodium glycocholate was accompanied by increasing difficulty in extracting the carotene into petroleum ether, so that it was necessary to add a greater amount of alcohol than usual. In each case sufficient alcohol was added to effect complete extraction of the colouring matter from the aqueous phase.

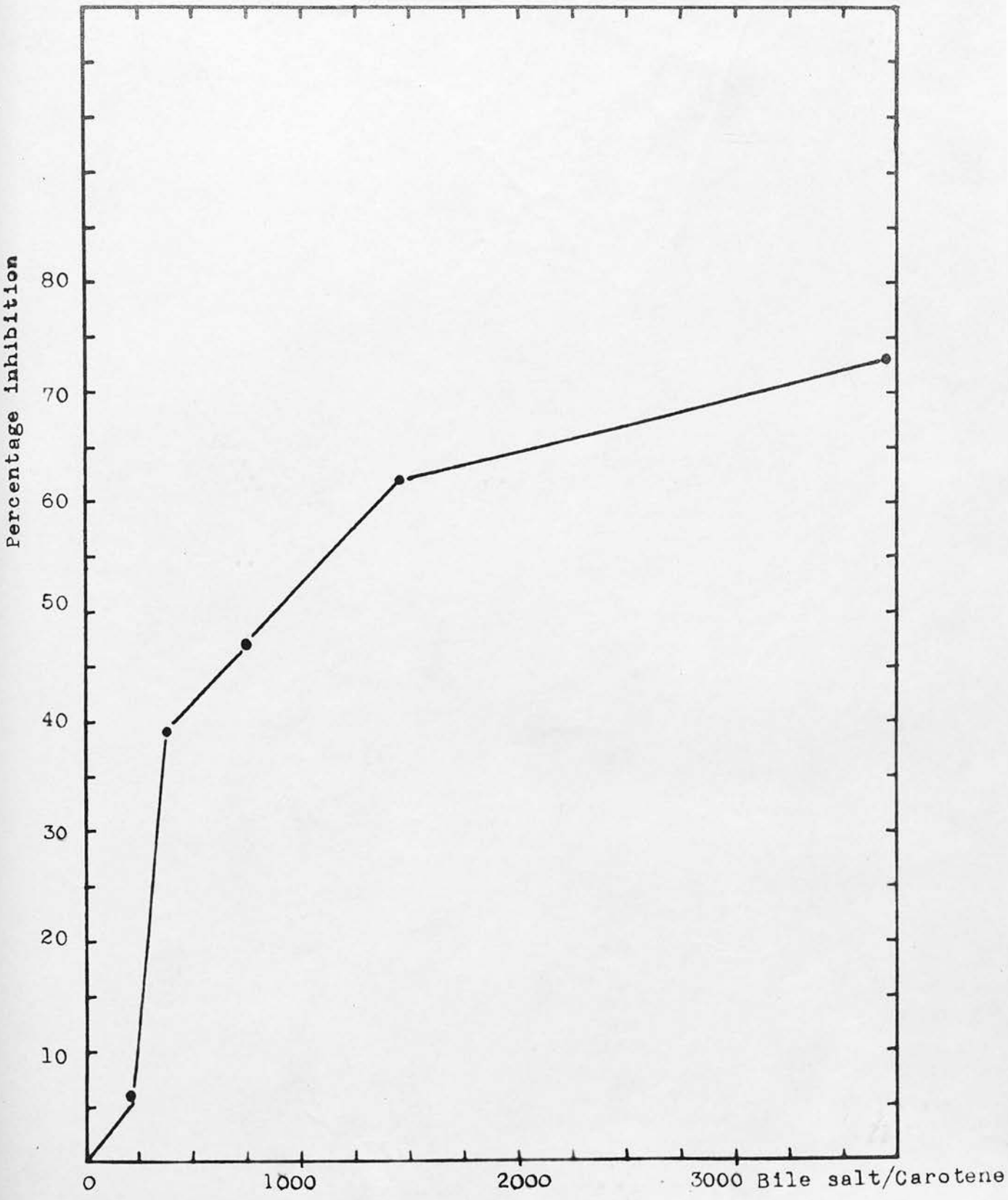

Fig.V. Percentage inhibition of oxidation of carotene with increasing concentration of bile salt.

e) Effect of Glycine, Alanine and Urea on the Oxidation of Carotene.

According to Karrer, dl-alanine exerts an accelerating power on the oxidation of carotene in colloidal aqueous solution. There is no information concerning the effect of the other amino acids or of urea. TableXIII shows the results of an experiment designed to compare the influence of molecular proportions of glycine, alanine and urea on the rate of oxidation of carotene. The molecular proportion of each of the three substances to carotene was 11,910 : 1. In that proportion the effect of the substances was so slight as to be negligible.

This experiment was repeated with larger amounts of urea, glycine and alanine, and a more concentrated solution of carotene (See Table XIV).

Again there was but slight acceleration of oxidation in the tubes containing urea and alanine and a negligible difference in the one containing glycine, even though the molecular proportions compared with that of carotene were very high. Comparison of the figures for tubes B. and C. shows that this slight effect is not quantitative. It seems that

TABLE XIII

HOURS	TUBE A	TUBE B	TUBE C	TUBE D
	γ per 5 ccs. colloidal solution	γ per 5 ccs. colloidal solution	γ per 5 ccs. solloidal solution	γ per 5 ccs. colloidal solution
0	9.0	9.0	9.0	9.0
1	5.8	4.8	5.3	4.8
2.25	2.8	2.0	3.0	2.0
4	1.0	0.5	1.3	0.9

Tube A contained 20 ccs. carotene solution. (CONTROL).
Tube B " " " " " plus 60 mg. urea.
Tube C " " " " " plus 75 mg. glycine.
Tube D " " " " " plus 89 mg. dl-alanine.

TABLE XIV

HOURS	TUBE A	TUBE B	TUBE C	TUBE D	TUBE E
	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution
0	37.0	37.0	37.0	37.0	37.0
2	32.0	28.5	26.0	29.5	28.5
4.5	23.0	20.3	20.0	21.8	17.0
6.5	16.3	15.3	15.1	15.9	15.0
22	8.5	4.0	4.3	6.3	4.5
46	0.8	0.5	0.7	0.8	0.5

		Molecular Proportion.
Tube A contained 30 ccs. carotene solution.	(CONTROL).	
Tube B " " " " "	plus 600 mg. urea.	24145
Tube C " " " " "	plus 1200 mg. urea.	48290
Tube D " " " " "	plus 750 mg. glycine.	24145
Tube E " " " " "	plus 890 mg. dl-alanine.	24145

serum carotene is unaffected by the presence of urea, glycine and alanine.

Fleischmann and Kann (11) have reported that thyroxine, di-iodotyrosine, tyrosine and adrenaline inhibit the autoxidation of vitamin A (in aqueous suspension) in contrast to similar substances without a phenol group, e.g. phenylalanine. These authors consider that the oxidation-inhibiting power of these substances is a function of their properties as phenol drivatives and is in agreement with the stabilising action of hydroquinones on vitamin A. To test this effect in the case of carotene, equimolecular proportions of phenylalanine, tyrosine and l-thyroxine were added separately to samples of a colloidal carotene solution, and the progress of oxidation was followed as usual. No significant effect was observed for the amounts used, which were small owing to the fact that very little l-thyroxine was available (molecular proportion of carotene to each of the amino-acids was 2:13). It was not possible to test the effect of adrenaline since free adrenaline is unstable in solution and adrenaline hydrochloride is sufficiently acid to cause precipitation of the colloid.

f) The Influence of the Serum Proteins.

The modifying effect of albumin on the properties of carotene in colloidal aqueous solution has been commented on by Karrer (see Introduction), and from his observations, it seems probable that serum carotene is profoundly influenced by the presence of serum proteins, both in its solubility properties and in its rate of oxidation. It was proposed, therefore, to investigate the oxidation-inhibiting power of pure serum albumin and globulin. For this purpose the proteins were required salt-free and fat-free and were prepared from plasma (from citrated blood) by the following method.

Preparation of Serum Albumin and Serum Globulin.

To one litre of plasma from citrated blood was added, slowly with constant stirring, one litre of saturated ammonium sulphate solution. A little toluene was added to prevent bacterial decomposition and the globulin was allowed to settle out overnight and then filtered off. The precipitate (globulin) was dissolved in saline (about 200 ccs.), transferred to a collodion sac and dialysed for 24 hours against running tap-water, and then for a further 12 hours against distilled water. All operations were carried out at room temperature. The globulin precipitated out. The volume of the dialysate was reduced by distillation *in vacuo* to about 100 ccs., which was then transferred to two 250 cc. centrifuge bottles. 200 ccs. of alcohol and a few drops of glacial acetic acid were added to each with stirring. After centrifuging for one minute, the supernatant fluid was decanted and the precipitates

were mixed with a further 100 ccs. alcohol. The centrifuging and decanting was repeated and then the deposits were stirred up with ether (100 ccs. to each bottle), centrifuged and the supernatant liquid decanted. The whole deposit was transferred to one bottle and the treatment with ether was repeated once, and then the deposit was spread out well on the sides of the centrifuge bottle, placed in a vacuum desiccator and evacuated for about 30 minutes. Finally, the globulin was transferred to a Petri dish, ground to a fine powder and dried in a desiccator over conc. sulphuric acid overnight. This process, from the point of adding the alcohol, was carried out as rapidly as possible to minimise the risk of denaturation.

The globulin so prepared was a fine, bluish-white powder. It was evidently partially denaturated as it did not dissolve completely in normal saline, even after standing for several days.

After the globulin precipitated by half-saturation with ammonium sulphate had been removed by filtration, the filtrate was saturated with solid ammonium sulphate and the precipitated albumin filtered off. After the precipitate had been dissolved in the minimum of water, it was dialysed, precipitated with alcohol and dried with ether as described in the case of the globulin. The final product was a creamy-white powder, which dissolved in water after standing overnight.

The problem then presented itself of extracting the carotene from protein solutions by petroleum ether so that it could be estimated colorimetrically. As Karrer reported, in the presence of albumin, the pigment takes on the solubility properties of a chromoprotein and cannot be extracted by fat solvents, a fact which has been confirmed many times.

Addition of alcohol, by denaturing the protein, renders some of the pigment extractable, but recovery experiments showed that only about 50-60% of the pigment was extracted into the petroleum ether by the usual method (described previously). Addition of larger amounts of alcohol produced but slight improvement. Attempts were made to obtain 100% recovery by changing the pH of the solution, but they were not successful. At very low pH values (1-3) most of the pigment could be extracted, but the high acidity resulted in the formation of unmanageable emulsions.

Ultimately it was found that prolonged shaking, as described by Yudkin (12), was the solution of the problem. It was found advantageous to lower the pH to about 5 by the addition of one or two drops of glacial acetic acid (Mystowski et al, 1943, Personal Communication). Two extractions were made in each case, although Yudkin considers that only one is necessary.

5ccs. of the protein-containing carotene solution, 5 ccs. of alcohol and 10 ccs. of petroleum ether were placed in a boiling-tube. Two drops of glacial acetic acid were added. The tube was stoppered and shaken by hand for 10 minutes. After centrifuging for one minute, the petroleum ether layer was removed by pipette, a further 10 ccs. of that solvent

added to the tube and the process repeated. If emulsions formed which were not readily separated by centrifuging, a few drops of alcohol were added and the centrifuging was repeated; the emulsions then separated out without difficulty. The extracts were combined, reduced in volume to 10 ccs. and used for the colorimetric estimations. By this means, quantitative recovery of the pigment was accomplished.

Thus:

Amount of Carotene added.	Amount Recovered from Albumin Solution (7%)
23	22.3 ; 22.5
15	14.8 ; 14.5
29	28.2 ; 27.9

This method of extraction of the pigment from protein-containing solutions was used throughout the following experiments. It is convenient to mention at this point that extraction of the pigment from serum-containing solutions and from serum itself was accomplished in the same way, but that a further treatment was necessary to remove any bile pigments which had been dissolved by the petroleum ether (Mystowski et al). The extracts were shaken once with 3% sodium hydroxide solution and washed twice with distilled water. The bile pigments were dissolved out by the alkaline solution so that the yellow colour of the petroleum ether layer was then due only to the carotenes (the xanthophylls remain with the alcohol during the extraction). The results are all expressed as β - carotene.

As a preliminary experiment, the effect of albumin on the oxidation of carotene was compared with that of starch in order to determine whether the inhibitory power, if any, was an intrinsic property of the albumin or whether it was merely the protective action

of a lyophilic colloid, The results of two such experiments are set out in Table XV.

In both cases, there is quite a marked inhibition of oxidation in the presence of albumin, whereas starch actually has a slight accelerating effect. Both colloids carry a negative charge at the pH of the experiments (5-6). This observation suggests that albumin probably exercises its influence on carotene by virtue of its protein nature and not merely of its colloid nature.

To determine whether or not this inhibiting power was quantitative, albumin was added in progressively increasing concentration to carotene solution. From the results of the two experiments shown in Table XVI it is apparent that there is increasing inhibition of oxidation with increasing concentration of albumin, within the limits of the concentration used.

A comparison was then made between the inhibitory power of pure serum albumin and that of serum itself. To 15 cc. of a preparation of colloidal carotene was added 10 ccs. of water containing 0.7 gms. albumin, and to another 15 cc. was added 10 ccs. of

TABLE XV

	HOURS	TUBE A γ per 5ccs. colloidal solution	TUBE B γ per 5ccs. colloidal solution	TUBE C γ per 5ccs. colloidal solution
Expt. I	0	21.8	21.8	21.8
	2	13.7	15.6	11.3
	5	8.1	11.3	5.6
	22	1.9	5.0	1.3
	46	0.0	1.3	0.0
Expt. II	0	36.2	36.2	36.2
	2	23.1	23.8	21.9
	7	9.4	20.5	1.9
	22	2.5	6.3	0.0

Tube A contained 15 ccs. carotene solution + 10 ccs. water (CONTROL)
Tube B " " " " " + " " " containing 1 gm.albumin *
Tube C " " " " " + " " " " 1 gm. starch *

*Final concentration of 4 gms. per 100 cc. solution.

TABLE XVI

	HOURS	TUBE A	TUBE B	TUBE C	TUBE D	TUBE E
		γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution
Expt. I	0	73.6	-	-	73.6	73.6
	2	64.5	-	-	71.5	72.8
	5	55.5	-	-	65.0	66.8
	24	26.8	-	-	37.0	41.0
	72	0.0	-	-	10.0	13.8
Expt. II	0	26.2	26.2	26.2	26.2	26.2
	24	11.3	12.5	13.1	15.0	18.1
	48	2.5	7.5	7.5	12.5	15.0
	72	0.0	5.9	7.5	12.2	13.7
	96	-	2.6	5.0	8.8	8.7

												Final Concn. of Albumin.
Tube A	contained	15	ccs.	carotene	solution	+	10	ccs.	water	(CONTROL).		
Tube B	"	"	"	"	"	+	"	"	"	containing	0.05 gms	alb. 0.2 gms.%
Tube C	"	"	"	"	"	+	"	"	"	"	0.2 "	" 0.8 gms%
Tube D	"	"	"	"	"	+	"	"	"	"	0.5 "	" 2.0 gms.%
Tube E	"	"	"	"	"	+	"	"	"	"	1.0 "	" 4.0 gms%

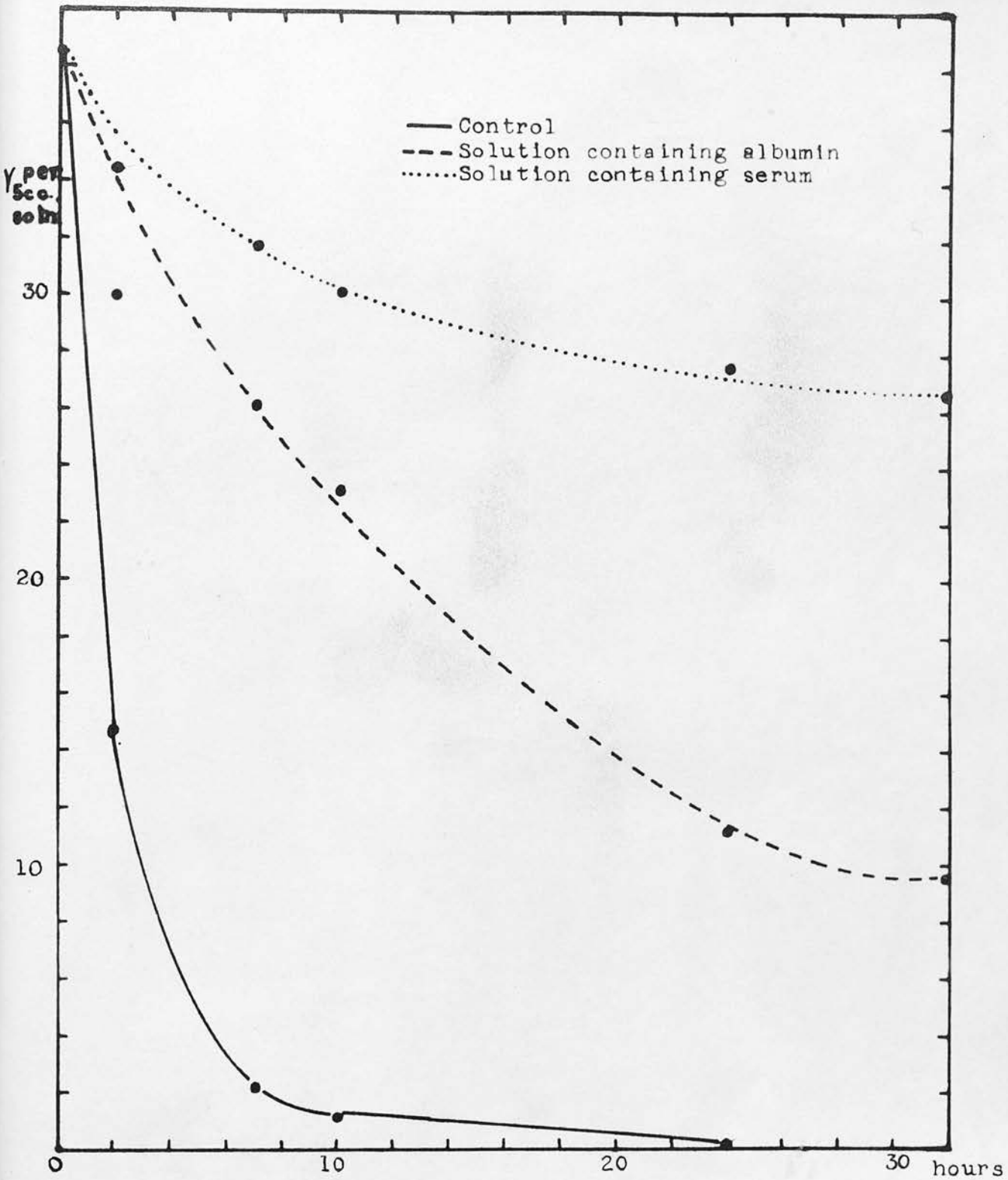

Fig.VI. The effect of pure serum albumin and of serum on the rate of oxidation of carotene in colloidal solution.

serum (equivalent to about 0.7 gms protein). The progress of oxidation of the carotene was followed as usual and compared with a control. At the same time, extracts were made from a tube containing 15 ccs. of water and 10 ccs. of serum, which was kept under the same conditions as the other tubes. The colorimetric readings of these extracts were subtracted from those of the extracts made from the serum-containing carotene solution, in order to allow for the pigment contributed by the serum itself. Fig. V1 shows that the inhibiting power of pure serum albumin, in a concentration corresponding to that of the total protein in serum, is less than that of serum itself.

Since it was possible that the superior inhibiting power of serum is due to its globulin content rather than to its albumin content, an experiment was carried out to contrast the effects of pure albumin, pure globulin and serum on the rate of bleaching of carotene. Since the globulin had to be dissolved in normal saline, saline was added to all the tubes in place of water, so that the conditions were exactly the same throughout (see Table XVII).

TABLE XVII

HOURS	TUBE A	TUBE B	TUBE C	TUBE D	TUBE E	D - E *
	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution	γ per 5ccs. colloidal solution
0	72.5	72.5	72.5	75.9	4.7	71.2
2	55.0	56.5	53.0	61.5	4.5	57.0
7	39.4	53.5	42.5	55.4	4.9	50.5
24	21.0	42.0	37.4	53.9	3.9	50.0
46	8.8	28.7	18.7	47.0	3.5	43.5

Tube A contained 15 ccs. carotene solution + 10 ccs. saline (CONTROL).
Tube B " " " " " + " " " containing 0.7 gms.albumin
Tube C " " " " " + " " " " 0.7 gms. globulin.
Tube D " " " " + 10 ccs. serum.
Tube E " 15 ccs. water + 10 ccs. serum.

*The figures in this column represent the progress of oxidation of the colloidal carotene actually added to the serum, discounting the colouring matter contributed by the serum itself. These figures, therefore, can be compared directly with those in columns A,B and C.

It is apparent that globulin exerts an oxidation-inhibiting effect on carotene similar to that of albumin, but it appears from this experiment that its effect is rather weaker than that of albumin. However, as has been mentioned previously, the globulin was partially denatured, a fact which may account for its reduced potency. The albumin, too, may have been partially denatured which would account for the result in Table XVI.

g) The Influence of lecithin.

Lecithin, according to Karrer, has a modifying effect on the state of colloidal carotene and diminishes its oxidation-rate. He gives no experimental results, however, and no information as to the source of lecithin.

A phosphatide preparation from brain was kindly presented by Dr. I.D.E. Storey, Department of Medical Chemistry. It had been preserved *in vacuo* for some time and was a pale-brown, flaky material. On analysis, it was found to contain 23.3% fatty acid and 43.6% of the theoretical amount of phosphorus (for lecithin), figures which suggest that this preparation consisted of approximately 40% lecithin, the remainder being made up of non-phospholipoid material.

Preliminary tests indicated that this material had a very marked inhibitory action on the oxidation of carotene and so further experiments were carried out to investigate the effect of varying concentrations. A colloidal solution of the "lecithin" was prepared by shaking the solid substance with water and allowing the suspension to stand for several hours with occasional shaking. Varying amounts

of this solution were taken and after their volumes had each been made up to 5 ccs. with distilled water, they were added separately to portions of a colloidal carotene solution. Aeration and periodic estimation of the carotene was carried out as usual. The results of two such experiments are shown in Table XVIII.

It is apparent from these results that the "lecithin" preparation had a distinct oxidation-inhibiting power over the carotene and that this power increased with the concentration of the "lecithin". Comparison with serum showed that this preparation in a concentration corresponding to that in serum (150-200 mg. per 100 cc.) had a considerably greater inhibitory power than that of serum itself when added to colloidal carotene solution, an observation which suggests that the potency of the preparation was due not to its lecithin content but to the unidentified impurity.

It was noticed in the course of these last experiments that there was greatly increased difficulty in extracting the carotene from the "lecithin"-containing colloidal solution into petroleum ether. Higher proportions of alcohol and more prolonged shaking had to be employed

TABLE XVIII

	HOURS	TUBE A	TUBE B	TUBE C	TUBE D	TUBE E
		γ per 5ccs. solution	γ per 5ccs. solution	γ per 5ccs. solution	γ per 5ccs. solution	γ per 5ccs. solution
Expt. I	0	59.0	59.0	-	59.0	59.0
	24	18.7	52.5	-	58.0	60.8
	28	15.6	-	-	-	-
	48	nil	9.4	-	13.7	16.3
	72	-	8.8	-	10.7	14.4
	96	-	nil	-	3.1	3.7
Expt. II	0	41.5	41.5	41.5	41.5	41.5
	18	32.5	38.0	39.4	40.0	42.2
	44	26.8	35.6	39.0	37.5	41.0
	64	7.5	32.2	36.2	36.2	35.6
	88	nil	30.0	32.2	30.0	36.2

Tube A contained 25 ccs. carotene solution + 5 ccs. water (CONTROL)
Tube B " " " " " + " " " containing 5 mg. "lecithin".
Tube C " " " " " + " " " " 10 mg. "
Tube D " " " " " + " " " " 20 mg. "
Tube E " " " " " + " " " " 50 mg. "

than was usual in the case of the non-protein-containing colloidal solutions. It seems probable that inhibition of oxidation and difficulty of extraction into fat solvents are closely interrelated (cf. serum carotene).

Another sample of lecithin, prepared from brain by Mr. L. Dzialoszynski, Dept. of Medical Chemistry, was used in a similar experiment. Decrease in the oxidation-rate of carotene was observed but it was not so marked as with the first "lecithin" preparation, nor was the same difficulty in extraction of the carotene experienced. This lecithin preparation showed, on analysis, the theoretical content of phosphorus. Unfortunately the supply was so small that no further investigations could be carried out, and very little significance can confidently be attached to the result of this one experiment.

CONCLUSION.

The findings of the foregoing experiments indicate that the rate of oxidation of artificial colloidal solutions of carotene is independent of the presence of ascorbic acid, glutathione, glucose, bile salts, alanine, glycine, urea, phenylalanine, tyrosine and thyroxine, unless these substances are present in concentrations many times those in which they are usually found in blood. It is reasonable to suppose, therefore, that serum carotene is unaffected by the presence in blood of these substances, and that its remarkable stability must be attributed to some other factor.

The work of Karrer suggests that the serum proteins and, to a lesser extent, the serum lipoids may be the agents responsible for the preservation of serum carotene, since they exert so profound an effect on the solubility properties of the pigment in artificial colloidal solution. It has been found in the present work that pure serum proteins have an inhibitory effect on the oxidation of carotene, a property not shown by another lyophilic colloid, starch. This

inhibitory effect, however, is considerably less than that of serum itself (when added to colloidal carotene solutions) and is certainly not comparable to the stabilising effect of serum on its natural carotene content. It must be emphasised at this point that serum does not preserve artificial colloidal solutions of carotene as efficiently as would be expected. The explanation of this must be that, in serum, the carotene is intimately associated with one or more of the other constituents of serum, probably the proteins, and that this intimate relationship is not brought about merely by adding protein or serum to carotene already in colloidal suspension.

Further work with pure samples of lecithin is required before any conclusion can be drawn as to the part played by it in the preservation of serum carotene. Since Karrer reports that the absorption spectrum of carotene changes if mixed with lecithin in alcoholic solution, whereas no change occurs if colloidal aqueous solutions of the two substances are mixed, it seems probable that an association is formed between the

two, and that this association requires the intimate mixing brought about by true solution (in alcohol). It would be advantageous to prepare the colloidal solutions in this manner, so that the lecithin would have greater opportunity for exerting its influence on the carotene.

It will be observed that the rate of oxidation of the carotene in the control tubes varies slightly in different experiments. This is assumed to be due to differences in size and dispersion of the colloidal particles, variable factors which depend on slight differences in the conditions during the preparation of the colloidal solutions. Physico-chemical factors are evidently of great importance, but consideration of them is beyond the scope of this paper.

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ACKNOWLEDGEMENTS.

I wish to express my thanks to the late Dr. E. M. Mystowski for suggesting this work, and to Dr. C.P. Stewart under whose direction it was carried out.

Part of the expenses were defrayed by a grant from the Moray Fund of Edinburgh University, for which I also wish to take this opportunity of expressing my thanks.

Reprinted from THE LANCET, *Nov.* 6, 1943, *p.* 571.

CONCENTRATION OF SULPHAPYRIDINE IN BILE

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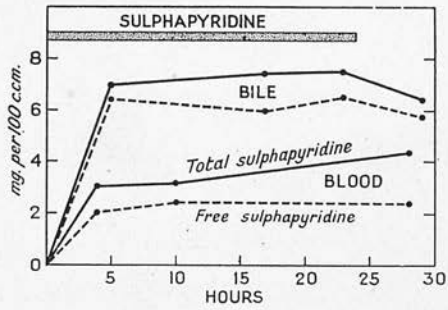
PUBLISHED data on the concentration of sulphonamide drugs in the bile are inconclusive and contradictory. Thus Bettman and Spier (1939) stated that, whereas with a normally functioning gall-bladder the concentration of sulphanilamide was always greater in the bile than in the blood, the reverse was true with a non-functioning gall-bladder. Spink and others (1941) claimed that the concentration of sulphanilamide was always lower in the bile than in the blood, although that of sulphapyridine was usually greater in the bile. But Hubbard and Butsch (1941), who used much smaller doses of the drug, found sulphapyridine to be in lower concentration in the bile than in the blood. Barber and co-workers (1943), in experiments on rabbits, found that in contrast to a new drug, sulphacholazine, which reaches a high concentration in bile after intravenous injection, sulphanilamide and sulphapyridine were never present in the bile except in very small amounts.

The experiments reported in this paper were made with the object of assessing the possible value of sulphapyridine as a biliary antiseptic. Patients on whom an operation for biliary fistula had been performed were the subjects. Some days were allowed to elapse after the operation before specimens were obtained, with the object of minimising the effects of the anæsthesia on the liver. After a control specimen of bile had been collected an initial dose of 2 g. of sulphapyridine was given by mouth, and thereafter a maintenance dose of 1 g. every four hours. Specimens of bile were taken for analysis at intervals of one to six hours from the time of the initial dose, and blood about every six hours for two days or longer. The analyses were carried out according to the method of Marshall



Level of sulphapyridine in blood and bile ; initial dose of 2 g. of the drug and thereafter 1 g. every four hours.

and Litchfield, and in the case of bile the pigments were removed by absorption on barium sulphate.

The findings are set out in the table. Of the eight cases studied all showed a higher maximal level in the bile than in the blood, usually about 6–8 mg. per 100 c.cm. in the bile when the blood concentration was about 3–4 mg. per 100 c.cm. In one case the bile sulphapyridine rose to 15 mg. per 100 c.cm. This was during the second day of the experiment. The drug had been given intramuscularly to avoid nausea and vomiting, and there was apparently considerable delay in absorption, as only traces were found in the bile and blood for twelve hours after the intramuscular injection.

A typical curve of the sulphapyridine concentration in the bile and blood is shown in the figure. In this case the biliary fistula had been made six days before the beginning of the investigation. The initial dose of 2 g. sulphapyridine was given by mouth at 11 AM, and thereafter 1 g. every four hours.

Two patients were given the above dose of sulphapyridine for twenty-four hours before cholecystectomy, and bile was taken from the common duct and gall-bladder at operation. In one case, the blood concentration was 4·5 mg. per 100 c.cm. (free) while the common-duct bile contained 8·0 mg. and the gall-bladder bile 11·7 mg. per 100 c.cm. free sulphapyridine. In the other case, the blood concentration was 3·1 mg. per 100 c.cm., while the common-duct bile contained 13·2 mg. free and the gall-bladder bile 12·9 mg. per 100 c.cm. free sulphapyridine (the surgeon reported that the patient had a non-functioning gall-bladder).

It may be concluded that despite the adverse effects on the secretory and concentrating power of the liver and gall-bladder which accompanied pathological changes in these organs. sulphapyridine may reach a concen-

LEVEL OF SULPHAPYRIDINE IN BLOOD AND BILE (MG. PER 100 C.CM.) IN 8 PATIENTS WITH BILIARY FISTULA

Case	Hours after initial dose	BLOOD		BILE	
		Free	Total	Free	Total
1	26	2·7	3·0	6·0	..
	32	4·0	4·7	15·5	..
2	15	..	..	6·4	6·6
	26	3·1	4·1	5·3	6·7
3	24	4·5	5·6	8·0	8·2
4	12	..	..	7·5	8·2
	26	3·1	3·6	9·2	10·4
5	3	2·5	2·8	5·1	5·9
	12	..	..	6·8	7·3
6	4	2·0	3·1	6·5	6·9
	30	2·2	4·8	5·7	6·2
7	24	3·1	3·8	12·2	13·0
8	24	2·2	2·7	9·0	11·5

In cases 1 and 2, sulphapyridine 2 g. was injected intramuscularly on two successive days.

In cases 3, 4, 5, 6, an initial dose of 2 g. sulphapyridine was given by mouth and thereafter 1 g. every four hours.

In cases 7, 8, specimens of blood and bile were taken at operation; 1 g. of sulphapyridine had been ingested every four hours for twenty-four hours before operation.

tration in the bile which, in blood, would be effective against certain organisms, and accordingly sulphapyridine appears to be worthy of clinical trial in infections of the biliary tract.

I wish to thank Prof. L. S. P. Davidson and Dr. C. P. Stewart, under whose direction this work was carried out, and the surgeons of the Royal Infirmary, particularly Prof. Learmonth, for their coöperation.

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